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CORRECTIONS

On page 733, Vol. 168, No. 2, May, 1947, throughout the article read α-D-*gluco-sido-α-L-ketoarabinoside* for α-D-*glucosido-β-L-ketoarabinoside*.

On page 585, line 1, Vol. 181, No. 2, December, 1949, read *The PIP fraction was separated from the RNA by the method of Delory (2). The DNA fraction was extracted from the RP by two 15 minute treatments with 5 per cent TCA at 90° for The PIP .fraction was extracted from the RP by two 15 minute treatments with 5 per cent TCA at 90°.*

SOME FACTORS AFFECTING THE POTENCIES OF VITAMIN B₁₂ AND LEUCONOSTOC CITROVORUM FACTOR OF CERTAIN NATURAL PRODUCTS*

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Considerable interest has developed in devising microbiological methods for estimating the potency of unknown factors in natural materials. Available reports indicate that *Lactobacillus leichmannii* 313 and 327 require vitamin B₁₂ (1–4) and that *Leuconostoc citrovorum* 8081 requires an unknown factor, apparently not vitamin B₁₂ (5, 6), also present in refined liver extracts.

In the present study the effect of heat, acid, and alkali treatment on the vitamin B₁₂ potency of vitamin B₁₂ concentrates, refined liver extracts, and animal tissues has been investigated with the use of *L. leichmannii* 313 and 327 as the test organisms. In addition, the relative stability to various treatments of the factor required by *L. citrovorum* has been studied. Preliminary studies on the enzymatic release of these factors have also been conducted.

EXPERIMENTAL

L. leichmannii 313 and 327 were maintained on a prepared medium of 100 parts of skimmed milk, 10 parts of neutralized tomato juice, and ½ part of yeast extract. Stock cultures of this medium were prepared weekly, incubated at 37° for 24 hours, and stored in the refrigerator. *Leuconostoc citrovorum* 8081 was maintained on a yeast-dextrose-agar medium fortified with additional B vitamins and peptone.

The basal medium used was essentially that described previously (7). In addition to purified amino acids, an enzymatic digest of casein (2) was added at a level of 6 mg. per tube and Tween 80 at the level of 0.01 ml. per tube for *L. leichmannii* 313 and 327 (1). In some experiments, acid-hydrolyzed casein plus the amounts of cystine, tryptophan, and asparagine

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described by Snell *et al.* (1) replaced the amino acid mixture. In later experiments cysteine hydrochloride was used in place of cystine, since much smaller amounts of cysteine are required for maximum growth of these organisms.[1]

Crystalline vitamin B_{12} was used as the standard and an injectable liver extract (15 U. S. P. units per ml.) was included in each assay as a secondary standard.

A similar basal medium (purified amino acids or a supplemented casein hydrolysate) was used for *L. citrovorum*. Folic acid was added to all the media at a level of 0.1 γ per tube. The enzymatic digest of casein and Tween 80 were omitted. A liver extract (15 U. S. P. units per ml.) was used throughout the experiments as the standard and was assigned a potency of 1 unit per ml. Vitamin B_{12} was found to be inactive for this organism, in agreement with the results of Lyman and Prescott (8).

TABLE I

Comparative Potencies* of Various Liver Extracts for Test Organisms

Source No.	Material tested	L. leich-mannii 313	L. leich-mannii 327	L. citro-vorum
1	Liver extract, 15 U. S. P. units per ml.	11.9	13.9	1.0†
2	" " 15 " " " "	3.0	3.0	4.0
3	" " 10 " " " "	1.6	1.5	1.5
3	" " 2 " " " "	0.79	0.77	0.4
	" " Wilson's 1:20	3.8	4.0	5.3

* The vitamin B_{12} potency is measured in micrograms per ml.

† Source 1 used as the standard and assigned a potency of 1 unit per ml.

All assays were carried out with a total volume of 10 ml. per tube. The tubes were autoclaved for 15 minutes at 15 pounds pressure and inoculated with 1 drop of inoculum (washed twice with 0.9 per cent saline solution). The medium used for preparing the inoculum from the stock cultures was the basal medium plus an excess of liver extract. Assay tubes were incubated for 72 hours at 37° and the acid production measured by titration with 0.1 N NaOH.

Selected samples of liver extract containing 15, 10, or 2 U. S. P. units per ml. and 1:20 liver extract powder were diluted and the potencies determined with all three organisms. Results of these studies are shown in Table I.

The vitamin B_{12} potencies of a vitamin B_{12} concentrate (approximately 70 per cent as active as the vitamin B_{12} standard), refined liver extract, beef liver, and beef round, as affected by heating with water, acid, or alkali, were

[1] Schweigert, B. S., Guthneck, B. T., and Scheid, H. E., unpublished data.

determined. 10 mμgm. of the vitamin B_{12} concentrate (in 5 ml. of distilled water) were autoclaved for 30 minutes with either 50 ml. of distilled water, 0.1 N HCl, or 0.1 N NaOH, the pH adjusted to 6.8, and the assay values compared with the untreated concentrate. 5 ml. aliquots of refined liver extract equivalent to 0.01 ml. of undiluted material (15 U. S. P. units per ml., Source 2) and 1 gm. samples of fresh beef liver were also treated as indicated above (Table II). *L. leichmannii* 313 was used as the assay organism in these tests.

The vitamin B_{12} potency of beef liver and round was also studied after .hot water extraction and was assayed with *L. leichmannii* 313 and *L.*

TABLE II

Vitamin B_{12} Potency of Various Materials As Influenced by Sample Treatment
L. Leichmannii 313 was used as the test organism.

Sample	Treatment	Vitamin B_{12} potency
		mμgm. per ml.
Vitamin B_{12} concentrate	None	7.0
	Autoclaved 30 min., H_2O	6.6
	" " " 0.1 N HCl	1.6
	" " " 0.1 " NaOH	<0.5
		γ per ml.
Injectable liver extract, 15 U. S. P. units per ml. (Source 2)	None	2.65
	Autoclaved 30 min., H_2O	2.48
	" " " 0.1 N HCl	<0.4
	" " " 0.1 " NaOH	<0.4
		γ per gm.
Beef liver	Cold H_2O extract	0.19
	Autoclaved 30 min., H_2O	0.33
	" " " 0.1 N HCl	0.069
	" " " 0.1 " NaOH	0.036

leichmannii 327. 1 gm. samples of beef liver or beef round were homogenized in a Waring blendor, treated with 50 ml. of water or acetate buffer (pH 4.5), autoclaved for 30 or 240 minutes, neutralized, and diluted to the appropriate volume for assay. The results of these studies are shown in Table III.

Studies on the liberation and stability of the factor required by *L. citrovorum* were conducted in a similar manner. The release of bound forms of the factors by pancreatic or ereptic digestion was conducted as follows: 1 gm. samples of beef and liver were homogenized in 50 ml. of water, 30 mg. of the enzyme added, adjusted to pH 7.0, preservative added (9), and the mixture incubated at 37° for 48 hours. At the end of the in-

cubation period the samples were checked for neutrality and diluted for
Results of the studies with *L. citrovorum* as the test organism (72

TABLE III

Effect of Sample Treatment on Vitamin B₁₂ Potency of Animal Tissues

Sample	Treatment	*L. leichmannii* 313	*L. leichmannii* 327
		γ per gm.	γ per gm.
Beef liver	Cold H₂O extract	0.22	0.27
	Autoclaved 30 min., H₂O	0.40	0.30
	" 240 " "	0.47	0.39
	" 30 " pH 4.5	0.32	0.22
Beef round	Cold H₂O extract	0.010	0.009
	Autoclaved 30 min., H₂O	0.009	0.015
	" 240 " "	0.012	
	" 30 " pH 4.5	0.015	0.014

TABLE IV

Liberation of Factor Required by Leuconostoc citrovorum

Material	Treatment	Potency in units per ml. or gm.*		
		Assay period		
		16 hrs.	*40 hrs.*	*72 hrs.*
Liver extract, 15 U. S. P. units per ml.	None	1.0	1.0	1.0
Liver extract, 10 U. S. P. units per ml.	"	1.27	1.47	1.73
Beef liver	Cold H₂O extract	1.19	1.40	1.03
	Autoclaved 30 min., H₂O	0.86	1.05	1.01
	Incubated 48 hrs.	1.96	2.00	3.28
	" 48 " with pancreatin	2.22	4.12	4.62
	Autoclaved 30 min., incubated 48 hrs. with pancreatin	1.28	1.67	0.80
Beef round	Cold H₂O extract	0.26	0.30	0.53
	Autoclaved 30 min., H₂O	0.13	0.14	0.13
	Incubated 48 hrs.	0.24	0.24	0.31
	" 48 " with pancreatin	0.71	1.06	0.86
	Autoclaved 30 min., incubated 48 hrs. with pancreatin	0.79	1.56	1.34

* Liver extract, 15 U. S. P. units per ml., used as a standard and assigned a
potency of 1 unit per ml.

hour incubation period) indicated that a considerable release of the bound
forms of the active factors occurred after enzymatic digestion.

In view of the fact that several factors, including thymidine and folic acid, may show activity with the *L. citrovorum* assay (1, 10, 11), further tests were carried out with a turbidimetric assay (16 and 40 hours incubation) and the results were compared with data obtained on the same sample preparations by the titrimetric procedure (72 hours incubation). Representative data obtained for these studies are presented in Table IV.

RESULTS AND DISCUSSION

The results obtained for the vitamin B_{12} potency of liver extracts indicate that assays with *L. leichmannii* 313 and 327 are measuring the same factor as shown by the consistency in the values for samples with a wide range of vitamin B_{12} potency (Table I). It seems probable that either organism can be used to measure the vitamin B_{12} potency of natural materials. It is recognized that the presence of thymidine or related substances would influence the assay values for vitamin B_{12}. Evidence is presented to show that *L. citrovorum* does not measure the vitamin B_{12} potency of these products. For example, the ratio of the vitamin B_{12} potencies of liver extracts, Source 1 as compared to Source 2, measured by *L. leichmannii* is approximately 4:1, while the ratio of the potencies of the same materials for *L. citrovorum* activity is 1:4.

A diluted vitamin B_{12} concentrate (Table II) was found to contain 7 mμgm. per ml. when compared to a vitamin B_{12} standard. After autoclaving for 30 minutes at 15 pounds pressure the value was found to be 6.6 mμgm. per ml., thus showing the stability of this factor to short term heating. The stability to heat is further substantiated by the results obtained for refined liver extract (2.65 γ per ml. vitamin B_{12} potency without heating and 2.48 γ per ml. for the same material autoclaved for 30 minutes at 15 pounds pressure). The vitamin B_{12} potency of beef liver autoclaved for 30 minutes was twice that obtained by cold H_2O extraction. Autoclaving for 30 minutes in 0.1 N HCl or 0.1 N NaOH reduced the vitamin B_{12} potency to approximately 20 per cent of the original potency. Lability of vitamin B_{12} to alkali has also been shown by Lyman and Prescott (8). Destruction by acid and alkali was also observed for injectable liver extract and beef liver.

The results in Table III show that the vitamin B_{12} potency of natural materials measured with either *L. leichmannii* 313 or 327 was not reduced appreciably by extended heat treatment or by heating at pH 4.5. This is further evidence for the similar specificity exhibited by the test organisms.

Studies conducted by autoclaving liver extracts, beef liver, or beef round with water, 0.1 N HCl, or 0.1 N NaOH revealed that these treatments were ineffective as methods to increase the values obtained for the *L. citrovorum*

factor. In fact the values were reduced considerably by acid treatment, but much less by alkali treatment. The latter finding substantiates the observation that vitamin B_{12} and the *citrovorum* factor are not identical.

Variations in the sensitivity of the vitamin B_{12} assay have been observed from one series of experiments to the next. Extensive tests to elucidate the cause of these variations have thus far been inconclusive. It should be pointed out that all tests comparing the effects of sample treatment were conducted within a single experiment. Although the actual values varied somewhat from one assay to the next, the relative values were consistent.

A series of experiments has been conducted on the vitamin B_{12} potency of natural materials after enzymatic treatment. Considerable difficulty was experienced in obtaining consistent results on repeated digests. The data indicate that a 2-fold increase in the vitamin B_{12} potency of beef liver was obtained after pancreatic digestion. In agreement with the report of Lyman and Prescott (8) the values for liver were higher than those for muscle.

The release of the factor required for *L. citrovorum* by pancreatin and erepsin was more consistent. A 2-fold increase was observed in the liberation of this factor from beef liver by autolysis or pancreatic treatment. A greater liberation from beef round than from beef liver was observed after enzyme treatment as compared to the results with cold water extraction. These results obtained both with the turbidimetric and titrimetric assays (Table IV) demonstrate the occurrence of bound forms of the factor capable of being released by enzymatic treatment, at least in part, to forms utilizable by the test organism. Further, the data show that a cold water extract of beef liver contains, in a microbiologically available form, more of the factor than comparable extracts of beef round. It will be noted that the potencies are somewhat higher for the enzymatic digests of liver, as measured by the titrimetric procedure (72 hour incubation), compared to the turbidimetric (16 hour incubation) procedure, suggesting some interference with the assay by thymidine and related factors when the longer incubation period was used. Nevertheless, release of the *citrovorum* factor occurred by enzymatic treatment as indicated by the relative values obtained for each incubation period of the assay studied. It is of interest that somewhat lower values were obtained for liver when the liver was autoclaved prior to pancreatic treatment. This indicates that autolytic enzymes in the fresh liver liberated the factor from either the liver or from bound forms of the factor in the pancreatin added. No decrease in the release of the *citrovorum* factor from beef round, attributable to autoclaving the samples prior to pancreatic treatment, was evident. The samples of pancreatin and erepsin used were found to be excellent sources of the

citrovorum factor (more than 1 unit of activity per gm.) and also of vitamin B_{12}.

The experience gained in these studies indicates that the complete release of vitamin B_{12} from natural materials is difficult. It appears likely that enzymatic techniques or simple hot water extractions offer considerable promise as a means of releasing bound forms of the vitamin in natural materials. As more experience is gained in the nutrition of the test organisms and on the comparative vitamin B_{12} potency of various products by animal assay (12), the specificity of the microbiological tests compared to the specificity for animals can be more adequately evaluated.

SUMMARY

The potencies of vitamin B_{12} measured by *Lactobacillus leichmannii* 313 and 327 and of the factor required by *Leuconostoc citrovorum* were determined in liver extracts, beef liver, and beef round. The potencies of these products were very similar when measured with *L. leichmannii* 313 and 327 as the test organisms. The potency of the factor required by *L. citrovorum* and the vitamin B_{12} potency of these materials varied independently.

The vitamin B_{12} potency of vitamin B_{12} concentrates, liver extracts, beef liver, and beef round was not reduced when these materials were autoclaved in distilled water for 30 minutes or 120 minutes. Approximately 80 per cent of the original activity was lost, however, when the samples were autoclaved for 30 minutes with 0.1 N HCl or 0.1 N NaOH.

Hot water extractions or the use of 0.1 N HCl or 0.1 N NaOH did not result in release of bound forms of the *citrovorum* factor from liver extracts, beef liver, or beef round. A reduction in potency was noted after acid treatment, while the values obtained after hot water or alkali treatment were essentially the same as for cold water extracts.

Hot water extraction resulted in some liberation of bound forms of vitamin B_{12}. Preliminary results indicate that enzymatic digestion results in a 2- to 3-fold increase in potency in the vitamin B_{12} and *citrovorum* factor of beef liver. Cold water extracts of beef liver contained more of the *citrovorum* factor in the microbiologically available form than comparable extracts of beef round. These studies with *L. citrovorum* were conducted with the use of both a 16 hour and a 72 hour incubation period for the assay.

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THE TRUE NATURE OF THE STIMULATION OF THE GROWTH OF LACTOBACILLUS ARABINOSUS 17-5 BY FOLIC ACID*

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It has been reported by Lewis (1), Isbell (2), and Snell and Mitchell (3) that p-aminobenzoic acid (PABA) is an essential growth factor for *Lactobacillus arabinosus* 17-5. Shankman *et al.* (4) did not find either PABA or folic acid (FA)[1] to be essential for this organism. Lampen and Jones (5, 6) reported that PABA, p-aminobenzoylglutamic acid (PABG), pteroic acid (PA), FA, and pteroyltriglutamic acid promote the growth of *L. arabinosus.* Of these factors PABA was stated to be most active. These authors believed that PABA, PA, and PABG are converted to FA or its higher conjugate, but found it puzzling that FA is less active than PABA in supporting the growth of this organism. Zalokar (7) found only very slight stimulation by FA of the growth of a PABA mutant strain of *Neurospora.* This stimulation was intimated to be due to the possible derivation of PABA from FA.

In this study data are presented to show that FA *per se* does not support the growth of *L. arabinosus.* The growth occurring in a solution of FA is found to be related to its decomposition products which may be, among others, PABG and PABA.

EXPERIMENTAL

A stock culture of *L. arabinosus* 17-5 was kindly supplied by Dr. D. J. O'Kane, Department of Botany. It was maintained in agar stabs prepared according to Morton and Engley (8) but containing a mixture of 0.1 γ of PABA and 0.1 γ of FA per ml. of medium. Transfers were made every 6 months and kept in the refrigerator.

Inocula—The inocula were prepared from the second subculture from the stock in the medium described below, to which 0.05 γ of PABA per ml. was added. The growth in the second subculture was harvested while still in the log phase (Klett-Summerson turbidity reading of 90 to 140 with a No. 54 filter). The cells were harvested and washed twice in M/15 phosphate buffer of pH 7.3 by centrifugation and then resuspended in this phosphate. The turbidity of the suspension was adjusted to a Klett reading of 60 and then diluted 1:100. To 10 ml. of growth medium, 0.1 ml. of this was delivered as an inoculum.

This inoculum represents about 30,000 cells per ml. of medium. When this routine was followed, no growth of *L. arabinosus* was obtained unless the medium was supplemented by PABA or other growth factors mentioned above. This enabled us to establish carefully the growth-stimulatory values for the factors studied here.

Medium—The composition of the medium used in this study is essentially that used for the growth of lactic acid bacteria. Since the quantities of certain components we used differ, the details of our medium are given in Table I.

To maintain the pH of the medium at neutrality throughout the growth period in some experiments, the medium was altered by the addition of 3 gm. per cent of phosphate buffer of pH 6.7 and the glucose concentration was lowered from 1 to 0.5 per cent to reduce the amount of acids formed.

Folic Acid Decomposition—The preservation of folic acid in a solution was one of the troublesome problems faced at the beginning of this study. Since there was no information available regarding this question, we carried out a special study to obtain a stable solution of FA. As we reported (9), solutions of FA in phosphate buffer, water, or bacterial growth medium decompose on standing at 30°.

We also found that FA decomposes in a refrigerator. Stokstad *et al.* (10) have reported on the effect of light, but in our experiments the solutions were kept in a dark incubator used exclusively for these experiments. Decomposition occurs in the presence or absence of growth. In an experiment, a solution of 200 γ of FA per ml. of growth medium was sterilized by filtration. At 0 hour the amount of diazotizable amine calculated as PABG was 7.64 γ per mg. of FA. After standing in the incubator for 22 hours the solution contained 16.26 γ of PABG per mg. of FA. When the conditions were such that growth of *L. arabinosus* 17-5 for a period of 22 hours yielded a turbidity reading of 214, the diazotizable amine as PABG was 18 γ per mg. of FA.

Solutions of FA in neutral phosphate buffer, bacterial medium, or normal rabbit serum were found to undergo very marked decomposition during a period of 10 to 20 days. Pteroyltriglutamic acid underwent similar

decomposition. The initial decomposition of FA solutions is at constant rate, which is followed by a short period of rapid decomposition.

Stabilization of Solutions of FA—A given solution of FA was best stabilized by keeping it in a frozen state in a dry ice freezer. For uniform testing, aliquots of a solution were kept in this freezer and one aliquot at a time was used for each experiment and the unused portions discarded. In this manner, we had a source of fresh solutions of known FA concen-

TABLE I

Composition of Medium Used for Growth of L. arabinosus 17-5

Solution I			Solution III		
Casein hydrolysate (vitamin-free)	10.00	gm.	$NaC_2H_3O_2 \cdot 3H_2O$	12	gm.
L-Cystine	0.20	"	H_2O (distilled)	50	ml.
Adenine sulfate	0.02	"	Solution IV		
Guanine hydrochloride	0.02	"	K_2HPO_4	5	gm.
Xanthine	0.02	"	H_2O (distilled)	50	ml.
Uracil	0.02	"	Solution V		
$MgSO_4 \cdot 7H_2O$	0.40	"			
NaCl	0.02	"	L-Tryptophan	100	mg.
$FeSO_4 \cdot 7H_2O$	0.02	"	Thiamine hydrochloride	200	γ
$MnSO_4 \cdot 4H_2O$	0.02	"	Calcium pantothenate	200	"
Pyridoxine	200	γ	Riboflavine	400	"
H_2O (distilled)	800	ml.	Nicotinamide	200	"
Solution II			Biotin	0.4	"
Glucose	20	gm.	H_2O (distilled)	50	ml.
H_2O (distilled)	50	ml.			

Solutions I, II, III, and IV are autoclaved in separate containers at 10 pounds pressure for 10 minutes to prevent caramelization. Solution V is filtered sterile through a sintered glass bacterial filter (UF) to prevent the destruction of the components. The aseptic combination of these solutions results in 1 liter of double strength medium. The final pH of this medium is 6.6 to 6.8.

tration and known diazotizable amine concentration prior to setting up growth experiments.

Fresh FA Solution—The amount of the diazotizable amine (or amines) calculated, for example, as PABG was 7.0 γ, or 3.5 γ of PABA per 1000 γ of FA.

Aged FA Solution—This represents an FA solution in M/15 phosphate buffer of pH 7.3 incubated in the dark in a 30° incubator for 10 days and taken out only during sampling to follow the increase in the amount of the diazotizable substances. This solution showed about 80 per cent loss of FA activity determined microbiologically with *Streptococcus faecalis* R

as the test organism.   The amount of the diazotizable amine calculated as PABG was about 60 to 65 per cent of the theoretical value and there was no increase in this respect on aging for longer periods.

Results

Effect of Aging of FA Solution on Its Ability to Stimulate Growth of L. arabinosus 17-5—Preliminary studies in this laboratory indicated a relationship between the degree of decomposition measured by the amount of
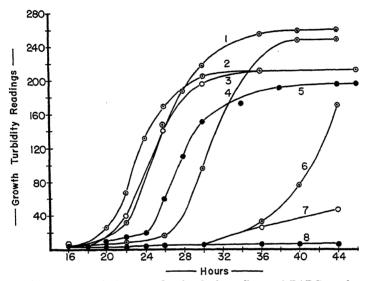
FIG. 1. Comparison of the growth-stimulating effects of PABG, and aged and fresh FA solutions on the growth of *L. arabinosus* 17-5.   Curve 1, 0.05 to 0.005 γ of aged FA; Curve 2, 0.01 γ of FA in medium aged for 48 hours before inoculation; Curve 3, 0.003 γ of PABG; Curve 4, 0.0005 γ of aged FA; Curve 5, 0.01 γ of FA in medium inoculated without aging; Curve 6, 0.00005 γ of aged FA; Curve 7, 0.00003 γ of PABG; and Curve 8, 0.0005 γ of fresh FA solution.   Quantities per ml. of medium.

diazotizable amine formed and the ability to stimulate the growth of *L. arabinosus*.   Two standard sets of media of identical composition were prepared.   FA was added to one set and the system was incubated for 48 hours at 30° before inoculation.   At this period, FA was added to the other set and both sets were simultaneously inoculated from the same bacterial suspension.   The curves in Fig. 1 serve as representatives of the results of many experiments performed as outlined above.   They show that the FA medium which was aged for 48 hours prior to inoculation (Curve 2) exercised a markedly greater stimulatory effect than the FA medium which was inoculated without aging (Curve 5).   In growth rate,

the latter system was 5 to 7 hours behind the former.   Since we have shown
(9) that a solution of FA aging for 22 to 48 hours undergoes from 1 to 2
per cent decomposition, the difference in the amounts of the active prod-
ucts formed in the two systems would appear to be responsible for the
difference in the rates of the stimulation of growth.

If the stimulatory effect of FA solutions is due to its decomposition
products, it would be expected that the greater the degree of decomposi-
tion the greater will be the difference between a completely aged and a
fresh FA solution in its effect on growth.   The curves presented in Fig.
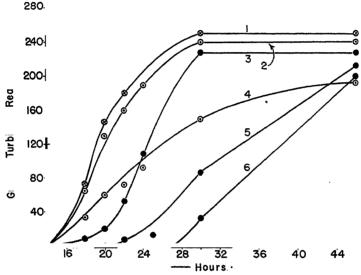
FIG. 2. Comparison of the growth-stimulating effects of PABA and fresh FA on
the growth of *L. arabinosus* 17-5.   Curve 1, 0.05 γ of PABA; Curve 2, 0.0005 γ of
PABA; Curve 3, 0.1 γ of fresh FA; Curve 4, 0.00005 γ of PABA; Curve 5, 0.01 γ of
fresh FA; and Curve 6, 0.005 γ of fresh FA.   Quantities per ml. of medium.

1 show that mole per mole aged FA solution (Curves 1, 4, and 6) is almost
100-fold more active than fresh FA solution (Curves 5 and 8).   It will be
seen that while 0.0005 γ of fresh FA (Curve 8) fails completely to support
growth 0.00005 γ of aged FA solution (Curve 6) produces an active growth
which, at the end of a 44 hour period, is at least 64 per cent of the maxi-
mal growth.

*Comparison of Growth Stimulation by PABA, PABG, Aged FA, and Fresh
FA.*—Since 80 per cent decomposed FA (aged FA) is almost 100-fold more
active than the solutions of fresh FA, the activities of PABA, PABG, and
the two FA solutions were compared.   The data presented in Fig.
2 (Curves 4 and 5) show that 0.00005 γ of PABA exercises much greater

stimulatory effect than 0.01 γ of fresh FA; PABA is more than 200-fold more active than fresh FA. A comparison of the growth curves further shows that the stimulatory effects of FA are associated with long induction periods, which would indicate that FA must undergo certain changes, for instance decomposition, before it can exercise stimulatory effects. The curves obtained with PABA, PABG, and aged FA are different from those of fresh FA in this respect. As shown by the plotted curves in Figs. 1
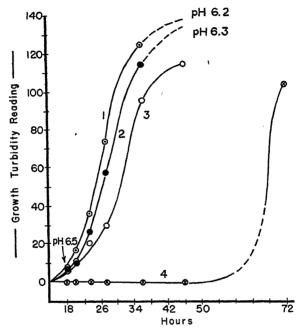
FIG. 3. Contrast between the growth-stimulating effects of PABA, PABG, aged FA, and fresh FA on the growth of *L. arabinosus* 17-5 in systems containing 3.0 gm. per cent of phosphate buffer of pH 6.7. Curve 1, 0.01 γ of PABA; Curve 2, 0.01 γ of aged FA; Curve 3, 0.01 γ of PABG; and Curve 4, 0.01 γ of fresh FA. Quantities per ml. of medium.

and 2, it can safely be stated that in their ability to support the growth of *L. arabinosus* 17-5 PABA and aged FA are the most active, followed by PABG, while fresh FA is the least active. In solution FA fails to maintain its molecular integrity. On the basis of the above quantitative comparisons, it could be concluded that FA is devoid of growth-supporting activity for *L. arabinosus* 17-5. Its growth stimulation is due to the decomposition products, among which PABA and PABG have been shown to occur (10).

Another striking difference between FA and its decomposition products is shown by the curves in Fig. 3. As described in the experimental sec-

tion, the pH of the medium was maintained at 6.2 to 6.6 during the entire growth period by altering the medium by the addition of 3.0 gm. per cent of phosphate buffer of pH 6.7. Under these conditions PABA, PABG, and aged FA (Curves 1, 2, and 3) exercised their growth-supporting activity during the usual period of 40 hours. In contrast, fresh FA solution (Curve 4) failed to stimulate growth for 46 hours. Only at the end of 72 hours was a partial growth observed.

The idea (6) that the utilization of PABA and PABG for the synthesis of FA accounts for the growth activity of FA solutions for *L. arabinosis* 17-5 must be reversed to mean that the decomposition of FA appears to be a requisite condition to account for its growth-supporting activity.

SUMMARY

The true nature of the activity of folic acid as a growth factor for *Lactobacillus arabinosus* 17-5 was investigated. A growth procedure so refined that no growth occurs without a supplement was used, which permitted a quantitative comparison of the growth-supporting activities of PABA, PABG, aged FA, and fresh FA solutions. .

In solution FA is not a stable molecular entity. It decomposes, yielding diazotizable amine (or amines) and other growth factors. An aged FA solution which had undergone 80 per cent decomposition (or 80 per cent loss of growth-supporting activity for *Streptococcus faecalis* R) was almost 100-fold more active than a freshly prepared solution of FA for the growth of *L. arabinosus* 17-5. PABA, or PABG, is likewise at least 100-fold more active than the solution of fresh FA. These data show that not the synthesis of FA from PABA, PABG, etc., but the disintegration of FA to the latter factors is essential for the stimulation of the growth of *L. arabinosus* 17-5.

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FAILURE OF FOLIC ACID TO ANTAGONIZE SULFANILAMIDE NON-COMPETITIVELY IN THE GROWTH OF LACTOBACILLUS ARABINOSUS 17-5*

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It has been postulated by Lampen and Jones (1) that, though less potent than p-aminobenzoic acid (PABA), folic acid (FA) is a growth factor for *Lactobacillus arabinosus* 17-5, and that PABA and p-aminobenzoylglutamic acid (PABG) indirectly are growth stimulants for this organism because they are utilized for the synthesis of FA. It was shown by us in the preceding paper (2) that (*a*) the aged FA solution which is 80 per cent decomposed is almost 100-fold more active than the solution of intact FA; (*b*) PABA and PABG are likewise at least 100-fold more active than the intact FA; and (*c*) when the pH of the medium is maintained at 6.2 to 6.6 during growth, the aged FA, PABA, and PABG complete their growth stimulation during a period of less than 40 hours; in contrast, the stimulation of growth by the intact FA is conditioned by a 60 hour or longer period of incubation which, apparently, is required for the splitting of the inactive FA into an active form. These facts permitted the conclusion that FA *per se* is inactive and that its decomposition products or products derived therefrom support the growth of *L. arabinosus*.

It was also postulated (1) that sulfonamides block the synthesis of FA via PABA, and that growth in the presence of FA is not subject to inhibition by these drugs for the reason that, there being no need for a synthesis of FA, there does not exist a susceptible reaction site for sulfonamides to block. On this basis, FA was considered a non-competitive antagonist to sulfonamides. If this were so, the growth in the presence of an optimal amount of FA and a sulfonamide should be expected to run simultaneously parallel to, or coincide with, the growth in the absence of the drug. The data presented here show that sulfanilamide (SA) inhibits the growth of *L. arabinosus* in the presence of FA. However, whenever a reversal occurs, it occurs at a period very much later than the conclusion of the log

phase of the growth in the absence of sulfanilamide. It therefore follows that FA *per se* is not an antagonist.

EXPERIMENTAL

The experimental conditions are the same as those described previously. As in the previous paper (2), in the determination of the degree and rate of growth, one failed to observe the informative intermediary changes if the measurement was made merely at the end of a 24 or 48 hour period. Preliminary experiments convinced us of the necessity of observing the entire
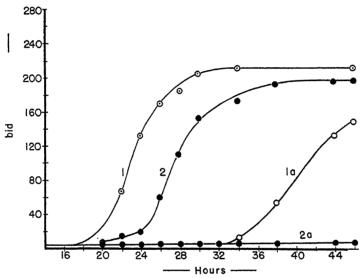
FIG. 1. Effect of aging on the antisulfanilamide activity of FA. Curve 1, 0.01 γ of FA aged for 48 hours before inoculation; Curve 1a, same as Curve 1 but containing 1 γ of SA; Curve 2, 0.01 γ of fresh FA per ml. of medium inoculated without aging; and Curve 2a, same as Curve 2 but containing 1 γ of SA.

growth curve to obtain precise information about the effects of various factors or combinations of factors.

Results

In our experiments, 0.05 to 0.1 γ of FA per ml. represents the optimal concentration to support the growth of *L. arabinosus*. A 10- to 100-fold increase in FA concentration (1.0 to 5 γ per ml.) does not appreciably increase either the rate or degree of growth. The data presented in Fig. 1 (Curves 2 and 2a) and Fig. 2 (Curves 3 and 3a) show that the growth supported by 0.01 γ of FA per ml. (85 per cent of the maximal) is completely inhibited by 1.0 γ of SA per ml. It will also be seen (Fig.

2, Curves 2 and 2a) that the growth supported by 0.1 γ of FA per ml. is inhibited by 1 γ of SA per ml. In this case, however, at a time when the drug-free system has reached the end of the log phase, the drug-containing system begins to show reversal of inhibition, which is complete after a period of 12 hours; a lag from the control of at least 12 hours persists at all time intervals. A disparity of at least 6 hours between the drug-free and drug-containing systems (Curves 1 and 1a) persists even in the presence of 5 γ of FA per ml. which is 100-fold in excess of the optimal growth requirement. In no case, therefore, do rates of the growth of *L. arabinosus*
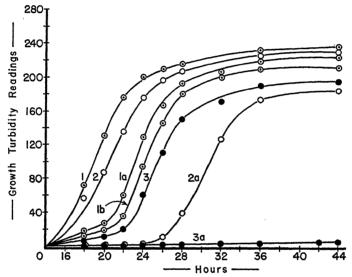
FIG. 2. Effect of concentration on the antisulfanilamide activity of fresh FA. Curve 1, 1 or 5 γ of fresh FA; Curve 1a, 5 γ of fresh FA containing 1 γ SA; Curve 1b, 1 γ of fresh FA containing 1 γ of SA; Curve 2, 0.1 γ of fresh FA; Curve 2a, same as Curve 2 but containing 1 γ of SA; Curve 3, 0.01 γ of fresh FA; and Curve 3a, same as Curve 3 but containing 1 γ of SA.

17-5 in SA-containing and SA-free systems run simultaneously parallel or coincide. The most critical argument against the concept that FA is a non-competitive antagonist to sulfanilamide is provided by the systems containing 0.01 γ of FA with and without 1 γ of SA per ml. Here, despite the fact that the growth in the SA-free system is 85 per cent of the optimal growth, FA is incapable of antagonizing the complete inhibition by sulfanilamide.

That the solutions of FA in sterile medium yield products which antagonize sulfanilamide is illustrated by the data presented in Fig. 1. Solutions of 0.01 γ of FA per ml. of medium which had been aged in the incu-

bator for 48 hours before inoculation manifested antisulfanilamide action at a very much earlier period than did the systems in which FA was not allowed to age before inoculation. Indeed, it is to be noted that 0.01 γ of FA per ml. of medium after aging for 48 hours showed antisulfanilamide action, while the corresponding system not subjected to aging failed to antagonize sulfanilamide (Fig. 1, Curve 2a, and Fig. 2, Curve 3a).

These observations are further corroborated by the data presented in Fig. 3 (Curves 1, 1a, 2, and 2a) which show that PABG and aged FA (80 per cent decomposed) antagonize 1 γ of SA per ml. 14 and 10 hours earlier,
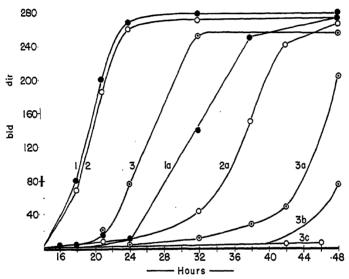
FIG. 3. A comparison of the antisulfanilamide activity of PABG, aged FA, and fresh FA. Curve 1, 0.01 γ of PABG; Curve 1a, same as Curve 1 but containing 1 γ of SA; Curve 2, 0.01 γ of aged FA; Curve 2a, same as Curve 2 but containing 1 γ of SA; Curve 3, 0.01 γ of fresh FA; Curve 3a, same as Curve 3 but containing 1 γ of SA; Curve 3b, containing 50 γ of SA, and Curve 3c, containing 500 γ of SA.

respectively, than the system containing fresh FA (Curve 3a). The latter system trails its respective control by a period of 20 hours. It will also be seen that fresh FA antagonizes 50 γ of SA per ml. only slightly (Curve 3b), while the complete inhibition by 500 γ of SA per ml. continues at the end of the 48 hour period (Curve 3c).

The gradual reversal by 0.05 to 5.0 γ of FA per ml. of the inhibition by 1 γ of SA per ml. can be related to the liberation of an adequate amount of decomposition products. As reported previously (2, 3), 1.0 to 5.0 γ of FA per ml. could yield 1 to 2 per cent diazotizable amine as PABG after an incubation period of 24 hours. The systems would therefore contain

0.01 to 0.05 γ of PABG per ml. to antagonize 1 γ of SA per ml. and to stimulate growth (see Fig. 3). In the presence of 0.1 γ of FA per ml. the concentration of PABG at the end of the 24 hour period would be about 0.001 to 0.002 γ per ml., which would cause only a partial reversal of the inhibition. This would permit partial growth, which is sufficient, as shown in Table I, to render the medium acidic and thereby render SA ineffective as inhibitor. As a consequence, a complete reversal of inhibition would follow.

Critical Effect of Changing pH of Medium during Growth on Sulfonamide Inhibition—The rate of the growth of a lactic acid bacterium is usually measured by titrating the amount of acids produced. Growth media used for this purpose have practically no buffering capacity. The medium used

TABLE I

Change of pH of Medium during Growth of L. arabinosus 17-5

0.25 per cent phosphate medium + 1 per cent glucose.

Growth medium containing	0 hr.	14 hrs.		16 hrs.		18 hrs.		20 hrs.		22 hrs.		24 hrs.	
	pH	T.*	pH	T.	pH	T.	pH	T.	pH	T.	pH	T.	pH
0.01 γ of PABA per ml.	6.6	2	6.5	9	6.1	43	5.6	81	4.9	135	4.5		
0.01 " " " +1 γ of SA per ml.	6.6	0	6.5	1	6.4	2	6.2	8	6.0	24	5.7	51	5.0
0.01 " " FA per ml.	6.6	0	6.5	1	6.5	3	6.4	11	6.0	28	5.8	69	5.0
0.01 " " " + 1 γ of SA per ml.	6.6	0	6.6	0	6.5	0	6.5	2	6.4	5	6.1	19	5.7

* T., turbidity readings with the Klett-Summerson photoelectric colorimeter, No. 54 filter.

by Lampen and Jones (1), for example, contains only 0.1 gm. per cent of phosphate mixture of pH 6.6 to 6.8. This medium undergoes an increase in H^+ concentration before a measurable growth occurs. In view of the critical effect of pH on the degree of sulfanilamide inhibition, the relative rates of pH change and growth of *L. arabinosus* 17-5 in the medium (2) which contained a 2.5-fold greater amount of phosphate (0.25 gm. per cent) were investigated. The data are presented in Table I. It will be seen that, on the average, in an SA-containing system a turbidity reading of 2 indicates a change of pH from 6.6 to 6.3 and a reading of 7 indicates a change from pH 6.6 to 6.1. A turbidity of 2 corresponds to a 350-fold increase in the number of cells (an increase from 30,000 (inoculum) to 10,500,000 cells per ml. at the end of an 18 hour period). The very sparing growth which results from the metabolism of glucose in the presence of sulfanilamide is necessarily associated with an increase in the concentration of H^+. An increase in H^+ concentration would suppress the in-

hibitory activity of sulfanilamide and represent a condition in which a very much smaller amount of antagonists, PABA or PABG, etc., is required to abolish the inhibition. Schmelkes *et al.* (4), for example, reported that 20 mg. per cent of sulfanilamide inhibited the growth of *Escherichia coli*, at pH 7.6, 6.8, and 6.0, 95, 71, and 54 per cent, respectively. The inhibitions with 5 mg. per cent of sulfadiazine at pH 6.7, 5.7, and 4.6 were, respectively, 95, 89, and 17 per cent. Brueckner (5), in experiments with *Staphylococcus aureus*, observed that at pH 6.5 a 5-fold greater amount of
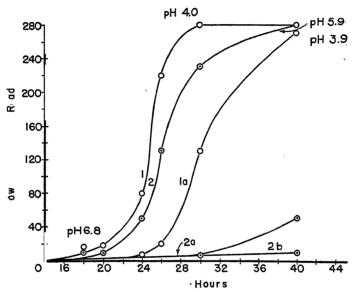
Fig. 4. Failure of FA to antagonize sulfanilamide in systems maintained at pH 6.0. Antisulfanilamide activity of FA in systems undergoing increment in H⁺ concentration. Curve 1, 0.05 γ of fresh FA in 0.25 gm. per cent of phosphate buffer; Curve 1a, same as Curve 1 but containing 100 γ of SA; Curve 2, 0.05 γ of fresh FA in 2.5 gm. per cent of phosphate buffer; Curve 2a, same as Curve 2 but containing 1 γ of SA; and Curve 2b, containing 100 γ of SA.

sulfanilamide is required than at pH 7.8, and that at pH 6.5 the SA:PABA ratio is 21,000 and 840 at pH 7.8. A 25-fold smaller amount of PABA is therefore required at pH 6.5 than at 7.8 to antagonize a given amount of SA.

These observations establish the fact that during growth a medium with very negligible buffering capacity will undergo a continued shift from neutrality to high acidity. This would both reduce the potency of sulfanilamide and raise the potency of the antagonist and thus readily abolish the inhibition of growth. It is therefore to be expected that if the medium is strongly buffered to prevent it from becoming acid in reaction the in-

hibition by sulfanilamide would present a basically different pattern.   Experiments were performed with systems containing an optimal concentration (0.05 γ per ml.) of FA in the presence of 0.25 and 2.5 gm. per cent of phosphate buffer.   The initial pH of these systems was 6.8.   The results of the experiment are presented in Fig. 4, Curves 1, 1a, 2, 2a, and 2b. Growth turbidities and pH values were determined at various intervals as shown in Fig. 4.   It will be seen that growth in 0.25 and 2.5 gm. per cent of phosphate (Curves 1 and 2) is nearly equal in degree and rate.   In
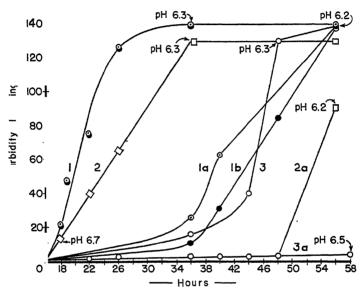
Fig. 5. A comparison of antisulfanilamide activities of PABA, PABG, aged FA, and fresh FA in systems containing 3 gm. per cent of phosphate buffer and 0.5 per cent glucose to maintain the pH at 6.2.   Curve 1, 0.01 γ of PABA or PABG; Curve 1a, 0.01 γ of PABA and 1 γ of SA; Curve 1b, 0.01 γ of PABG and 1 γ of SA; Curve 2, 0.01 γ of aged FA; Curve 2a, same as Curve 2 but containing 1 γ of SA; Curve 3, 0.01 γ of fresh FA; and Curve 3a, same as Curve 3 but containing 1 γ of SA.

the weakly buffered medium the pH had changed from 6.8 to 4.0 (Curves 1 and 1a).   In the system (Curve 1a) containing 100 γ of SA per ml. when growth had barely started (turbidity 20, 26 hour period) the pH had changed from 6.8 to about 5.0.   In this acid region sulfanilamide is ineffective.   At this period, in which growth lags behind that of the control by 6 hours, the growth curve, as would be expected, climbs steeply, and reversal is complete at the end of the 40 hour period, when the pH of the medium is 3.9.   In contrast, the pH of the reaction in 2.5 gm. per cent of phosphate medium (Curve 2) was 6.0 at the end of the 24 hour period and remained relatively constant throughout the entire growth period,

Under these conditions growth in the presence of 100 γ of SA per ml. of medium was completely inhibited (Curve 2b), and in 1 γ of SA per ml. at the end of the 40 hour growth period a partial reversal (18 per cent) was evident (Curve 2a).

In another experiment, the phosphate content of the medium was increased to 3.0 gm. per cent and the glucose content was reduced to 0.5 per cent to increase the buffering capacity of the medium and to reduce the production of acid. The pH of this medium at 0 hour was 6.7 and remained at a level of pH 6.2 to 6.3 throughout the growth periods. Since glucose was reduced from 1.0 to 0.5 per cent, the total growth was likewise reduced to one-half of the growth obtained in systems with 1 per cent glucose. The data are presented in Fig. 5. It will be seen that growth in the presence of 0.01 γ per ml. of PABA, PABG, or aged FA follows the usual trend (Curves 1 and 2). The growth in the system with fresh FA (Curve 3) lags behind that of the others by nearly 22 hours. In the presence of 1.0 γ of SA per ml. the inhibitions are antagonized by PABA and PABG (Curves 1a and 1b). Here growth curves lag behind those of the respective controls by about 20 hours. There was a 26 hour interval before antagonism occurred (Curve 2a) in the presence of aged FA (80 per cent decomposed). There was no sign of antagonism to 1.0 γ of SA per ml. by fresh FA at the end of the 58 hour period (Curve 3a). The data show again that when the pH of the medium is maintained at a constant level near neutrality throughout the growth period FA is incapable of acting as a non-competitive antagonist to sulfanilamide.

SUMMARY

Folic acid *per se* has been shown not to be a growth stimulant for *L. arabinosus* 17-5. Growth in the presence of FA follows a characteristic induction period corresponding to the time required for the liberation from FA of a sufficient amount of more active products such as PABG, etc. Folic acid *per se* has likewise been shown to be incapable of functioning as an antagonist to sulfanilamide. A delayed reversal of the inhibition of growth in systems with FA has been shown to be related to an inadequate buffering capacity of the medium. Rendering the medium acid would abolish the inhibitory power of sulfanilamide. In a strongly buffered medium complete inhibition persists without reversal. The postulate that sulfonamides interfere with the synthesis of folic acid via *p*-aminobenzoic acid is not supported by the experimental facts reported here.

The data derived here with *p*-aminobenzoic acid or *p*-aminobenzoylglutamic acid in experiments with *L. arabinosus* 17-5 do not permit correlation with data with organisms stimulated solely by FA.

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DETERMINATION OF MOLECULAR WEIGHTS OF PROTEINS IN SPREAD MONOLAYERS

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It is possible to determine the molecular weights of proteins by force-area measurements on spread monolayers at low film pressures (1–3). The method has the advantages of simplicity, speed, and economy of materials and apparatus.

The present investigation was undertaken to extend those studies and to define the conditions under which reliable results may be obtained. Force-area measurements at low film pressures under a variety of conditions have been made on films of the following proteins: hen's egg albumin, bovine serum albumin, rabbit serum γ-globulin, insulin, pepsin, chymotrypsinogen, chymotrypsin, and trypsin.

Theoretical

Bull reported an empirical equation of state for spread monolayers of proteins at film pressures below 1 dyne per cm. (2). This equation is

$$FA = \alpha F + \beta \tag{1}$$

where F is the film pressure expressed in dynes per cm., A is the area of the spread film in sq.m. per mg. α and β are constants into whose nature we propose to inquire.

If a_0 is the activity of the water underlying the surface, a the activity of water in the clean surface, then to expand the surface by transferring 1 mole of water from bulk into the surface at constant temperature and pressure the free energy required is evidently

$$\Delta F = A_1 \sigma_0 = RT \ln a/a_0 \tag{2}$$

where A_1 is the area occupied by 1 mole of water, σ_0 is the surface tension of water, R is the gas constant, and T is the absolute temperature. The corresponding free energy change for a surface containing n_2 moles of spread protein is

$$\Delta F_1 = A_1 \sigma_1 = RT \ln a_1/a_0 \tag{3}$$

where a_1 is the activity of the water in the surface containing the spread protein. Subtracting Equation 3 from Equation 2, we have

$$A_1(\sigma_0 - \sigma_1) = FA_1 = RT \ln a/a_1 \tag{4}$$

27

where F is the film pressure. Now a/a_1 is nearly equal to N/N_1 where N is the mole fraction of water in the clean surface and N_1 is the mole fraction of water in the surface containing spread protein. N is evidently unity and, accordingly, Equation 4 becomes

$$FA_1 = -RT \ln \left(1 - \frac{n_2}{n_1 + n_2}\right) \tag{5}$$

where n_1 is the number of moles of water in the surface on which protein has been spread. Since n_1 is very much greater than n_2, we have after expanding the logarithmic term and rearranging

$$n_1 A_1 F = n_2 RT \tag{6}$$

Evidently, $n_1 A_1$ is the area occupied by water and

$$n_1 A_1 = A - n_2 S_p \tag{7}$$

where A is the total area of the surface and S_p is the area per mole of protein. Substituting Equation 7 into Equation 6, we have, after rearranging,

$$FA = n_2 RT + n_2 S_p F \tag{8}$$

Equation 8 is identical in form with Equation 1 and by inspection, $n_2 S_p$ is equal to α and $n_2 RT$ is equal to β. At 25° β is, accordingly, equal to 24.6×10^2 per M, where M is the film molecular weight of the spread protein.

We see from the simple derivation outlined above that the pressure exerted by a spread film is closely analogous to an osmotic pressure and not to a gas pressure. Thus the term $n_2 S_p$ does not involve a collision diameter and is not equivalent to the b constant in van der Waals' equation; $n_2 S_p$ is a direct measure of the surface occupied by the spread protein.

Spread films of protein can show a considerable departure from Equation 8. Some of these departures will be noted in detail in the present paper. We have had occasion to study spread films of sodium lauryl sulfate, of the antibiotic, bacitracin, and of adrenotropic cortical hormone. While we do not intend to report on these substances at the present time, it is perhaps appropriate to note that the spread films of these three substances show a rather pronounced minimum in the FA versus F plots. We attribute this minimum to association of the spread molecules on the surface as the film is compressed. So far no other substances which we have studied have shown a minimum in the FA versus F plot.

EXPERIMENTAL

The Wilhelmy slide method and the float type of balance were used to measure the film pressures.

The Wilhelmy slide consisted of two vertical and parallel microscope cover-glasses 5 × 4.5 cm. suspended from an arm of a chainomatic analytical balance, dipping through the surface of the solution upon which spreading occurred. The microscope cover-glasses were No. 00 and were from 50 to 60 μ in thickness. They were obtained on special order from the Central Scientific Company of Chicago. An optical lever was attached to the balance and the deflection of the light beam produced by compression of the spread film measured. This deflection was calibrated in terms of weight added to the balance. It was possible to measure film pressures down to 0.01 dyne per cm. and to estimate pressures to 0.001 dyne per cm.

The float balance had no novel features except that there was no fixed attachment of the float to the sides of the trough. A fine silk thread dipped in a dilute solution of paraffin wax in benzene and allowed to dry was attached to the front of the float with paraffin wax. The thread extended about 2 cm. beyond each end of the float and simply lay against the edge of the trough. With proper care no leakage of the spread film occurred between the edge of the trough and the thread. The sensitivity of the balance was considerably increased by avoiding a fixed attachment of the float. An optical lever was used in connection with the float balance, and it was possible to read to 0.013 dyne per cm. and to estimate to 0.0013 dyne per cm.

The egg albumin was crystallized from fresh hen's eggs by the sodium sulfate method. Crystalline bovine serum albumin, crystalline pepsin, crystalline chymotrypsinogen, crystalline chymotrypsin, and crystalline insulin were obtained from Armour and Company. Serum γ-globulin was prepared from rabbits which had been sensitized against egg albumin. It was fractionated by sodium sulfate and gave a titer corresponding to about 80 per cent antibody. The crystalline trypsin was supplied by Dr. Hans Neurath.

A number of substrate solutions were used and considerable attention was paid to purity. The ammonium sulfate was recrystallized several times. The concentrated mother solution was treated with activated charcoal and filtered. Unless otherwise stated, a 5 per cent solution was employed as the substrate solution and was adjusted to pH 5.00. Water was distilled from alkaline permanganate. The glycerol was exhaustively extracted with petroleum ether and the petroleum ether removed completely. The hard clear paraffin wax which melted between 56–58° was refluxed with concentrated sodium hydroxide and then exhaustively extracted with water. Both balances were protected by cabinets. It was impossible to eliminate completely the surface-active impurities, and compression of the clean surface within 8 cm. of the end of the trough yielded

a pressure of about 0.01 dyne per cm. The trough was cast aluminum and was 60 cm. long and 14 cm. wide.

Results

The protein most thoroughly investigated was egg albumin. It was found that, when the initial surface concentration of this protein was less than about 0.30 mg. per sq.m., a break in the *FA versus F* plots occurred. This behavior is illustrated in Fig. 1.

<div style="text-align:center">Fig. 1   Fig. 2</div>
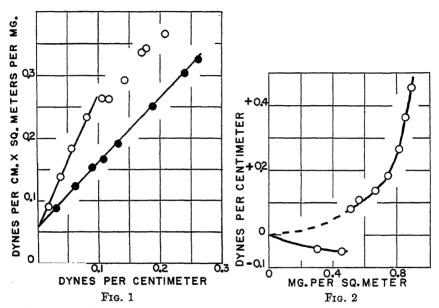
Fig. 1. Plot of *FA versus F* for egg albumin spread on 5 per cent ammonium sulfate solution.   O, 0.20 mg. per sq.m.; ●, 0.35 mg. per sq.m.   5 minutes elapsed time.

Fig. 2. Plot of film pressure against film concentration of egg albumin. 3 per cent ammonium sulfate containing 2 per cent glycerol. Wilhelmy balance. 3 minutes elapsed time.

The break in the *FA versus F* plot noted in Fig. 1 became progressively more pronounced as the initial surface concentration was decreased and the longer the film was allowed to remain on the surface before compression was started (referred to hereafter as the elapsed time). We believe that the protein molecules on the surface can exist in a compact form, which we shall call State B. Under appropriate conditions the compact spread protein molecules can expand to State A. There is, no doubt, a variety of intermediate expanded forms of the molecules and the transition from State A to State B is not, in general, sharp. We attribute the

break in the *FA versus F* plots to a transition of the expanded to the compact form upon compression, with a consequent change in value of S_p (see Equation 8). We shall discuss our reasons for this assumption presently.

In general, the results obtained with the Wilhelmy balance and those with the float balance were indistinguishable from each other, and in reporting our results we shall make no distinction between them. There was, however, one conspicuous exception to this good agreement. The Wilhelmy balance with dilute films of egg albumin and in the almost complete absence of surface-active impurities gave apparent negative pressures as the film was compressed. These pressures became progressively more negative on compression until quite suddenly, at a surface concentration of about 0.5 mg. per sq.m., the film pressure became positive and corresponded to what was to be normally expected at this compression. Shown in Fig. 2 is the film pressure plotted against the film concentration for such a film of egg albumin.

At first, we attributed the behavior shown in Fig. 2 to the displacement of surface-active impurities from the surface. However, more vigorous measures taken against the presence of these impurities only served to make the effect more consistent. Such negative pressures have not been observed with the float balance. We have come to the tentative conclusion that the negative pressures are due to the deposition of expanded egg albumin molecules (State A) on the glass slides. Such a deposition tends to be prevented by surface-active impurities. These deposited molecules are released when the egg albumin molecules revert to State B.

We have studied films of egg albumin spread on 5 per cent ammonium sulfate adjusted to pH values of 5.0, 4.08, and 3.60 with sulfuric acid. The initial spreading concentration of the protein was 0.35 mg. per sq.m. The *FA versus F* plots were all linear down to a film pressure of 0.01 dyne per cm., the limit of accurate experimental observation, and were indistinguishable from each other. The calculated molecular weight for these films was about 45,000 and the protein area was 1.02 sq.m. per mg. One spreading of egg albumin at an initial surface concentration of 0.17 mg. per sq.m. and on 1 per cent ammonium sulfate adjusted to pH 2.3 was made. There was a marked break in the *FA versus F* plot and the protein area was 2.15 sq.m. per mg. in the expanded State A. The molecular weight was about 45,000.

We have found 5 per cent magnesium sulfate to be an excellent substrate solution for the spreading of egg albumin. Thus, 0.30 mg. per sq.m. yielded a film whose molecular weight was about 45,000 and whose protein area was 0.89 sq.m. per mg. The *FA versus F* plot was linear.

The influence of previous heat denaturation in the acid as well as in the basic region on the spread films of egg albumin has been determined.

A 0.029 per cent solution of egg albumin was adjusted to pH 3.15 with dilute hydrochloric acid and heated on a boiling water bath for 15 minutes and then spread on a 5 per cent ammonium sulfate solution. The *FA versus F* plot was linear and corresponded to a molecular weight of about 45,000 and to a protein area of 1.05 sq.m. per mg. A 0.029 per cent solution of egg albumin was brought to pH 11.95 by the addition of sodium hydroxide and heated on a boiling water bath for 5 minutes. The *FA versus F* plot was again linear and corresponded to a molecular weight of about 45,000 and to a film area of 1.18 sq.m. per mg.

Altogether, the force-area relations of more than fifty egg albumin films have been studied under a variety of conditions during this investigation. All these films yielded a molecular weight of about 45,000, which agrees well with the molecular weight of this protein as determined by osmotic pressure and by the ultracentrifuge. The protein areas for the compact state (State B) were all in the neighborhood of 1 sq.m. per mg. The protein area for alkaline heat-denatured film noted above was the largest compact area observed (1.18 sq.m. per mg.). The protein area of 0.89 sq.m. per mg. observed on magnesium sulfate appears to be significantly smaller than that on ammonium sulfate. The areas corresponding to the expanded state (State A) were on occasion well in excess of 2 sq.m. per mg. The *FA versus F* plots were reversible provided the protein molecules had not expanded (State A). If initial expansion of the molecules had occurred, then expansion of the total area of the film after compression follows a *FA versus F* plot for a film in State B and not for State A.

Films of bovine serum albumin exhibit a break in the *FA versus F* plots, but unlike egg albumin films, there is no critical initial surface concentration at which expansion does not occur; the break in the *FA versus F* plot simply becomes more conspicuous the lower the initial surface concentration and the longer the elapsed time. When spread on 5 per cent ammonium sulfate solution, the *FA versus F* plot below about 0.1 dyne per cm. could be extrapolated to zero pressure without ambiguity to yield a molecular weight of about 70,000 and a protein area in excess of 2.0 sq.m. per mg. As the bovine serum albumin films were compressed to pressures above 0.3 dyne per cm., the *FA versus F* plot again became linear and could be extrapolated to zero pressure to yield a molecular weight of about 70,000 and a protein area of 1.2 sq.m. per mg.

Pepsin spread on 5 per cent ammonium sulfate solution yielded films which were associated, the degree of association being a function of the initial surface concentration of the protein. Thus at 0.19 mg. per sq.m., the molecular weight is calculated to be 56,000, while at an initial spreading concentration of 0.74 mg. per sq.m. the molecular weight is 88,000. Intermediate molecular weights were found for intermediate initial spreading concentrations. The *FA versus F* plots were all linear with no breaks.

The protein area was about 0.80 sq.m. per mg. for all of these films. However, if 2 per cent glycerol by volume was added to the ammonium sulfate solution, the degree of association of the pepsin was decreased and an initial spreading concentration of 0.19 mg. per sq.m. yielded a film molecular weight of about 35,000, in agreement with that previously reported (3). There was no break in the *FA versus F* plots.

No interaction could be observed when films of pepsin and of egg albumin were mixed on the surface of 5 per cent ammonium sulfate solution containing 2 per cent by volume of glycerol. Nor could any interaction be observed on the surface of 1 per cent ammonium sulfate solution adjusted to pH 2.3 with sulfuric acid. The compression of the combined films simply followed an *FA versus F* plot which could be predicted from a sum of the individual films. Thus pepsin films do not digest egg albumin films.

Insulin, when spread on 5 per cent ammonium sulfate solution, exhibited a slight tendency at low initial spreading concentrations (0.18 mg. per sq.m.) to expand and to give a break in the *FA versus F* plot, but even at 0.06 dyne per cm., the film had reverted to State B with a molecular weight of about 35,000 and a protein area of 1.03 sq.m. per mg. All higher initial spreading concentrations yielded completely linear plots. This behavior appeared to be independent of elapsed times from 1 to 5 minutes. However, when the ammonium sulfate solution was adjusted to pH 3.5 with sulfuric acid, the insulin film underwent such a great expansion that it was not possible to extrapolate the *FA versus F* to zero pressure with any confidence and, accordingly, we have no estimate of the film molecular weight of this protein under these conditions. When 2 per cent glycerol by volume was added to the 5 per cent ammonium sulfate solution at pH 5.0, an expansion of the insulin film was observed at all initial spreading concentrations tried (up to 0.75 mg. per sq.m.). The *FA versus F* plots gave two linear segments corresponding to State A and at higher pressures to State B. Both segments extrapolated to zero pressure to yield a molecular weight of about 35,000. The slope of the *FA versus F* plot at low pressure corresponded to a protein area of about 3 sq.m. per mg., while the slope at higher pressures gave a protein area of 0.98 sq.m. per mg. Fig. 3 shows the *FA versus F* plots for insulin films in the presence and absence of glycerol. Above about 0.35 dyne per cm., the values obtained in the presence of glycerol lie on the extrapolated line obtained without glycerol. This second segment is not shown in Fig. 3.

The behavior of the films of chymotrypsinogen and of chymotrypsin resembled each other very closely. Both proteins gave films on 5 per cent ammonium sulfate solution which yielded excellent linear plots of *FA versus F*. Elapsed time and initial spreading concentration had little or

no influence on the slope or on the intercept of these plots. Both proteins have, under these conditions, a film molecular weight of about ·80,000. The protein area for chymotrypsinogen was 1.07 sq.m. per mg., while the area for chymotrypsin was 0.85 sq.m. per mg.

When spread on 5 per cent ammonium sulfate solution containing 2 per cent glycerol by volume, the film molecular weight of both proteins was about 40,000. This molecular weight was independent of the elapsed time and of the initial spreading concentration (0.17 mg. per sq.m. to 0.68 mg. per sq.m.). Linear *FA versus F* plots were obtained. The slopes of
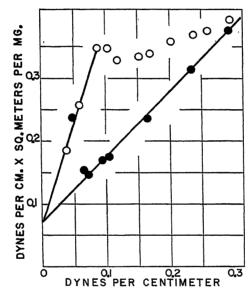
Fig. 3. Plot of *FA versus F* for insulin films on 5 per cent ammonium sulfate solution. Initial spreading concentration of 0.18 mg. per sq.m. ●, in absence of glycerol, 5 minutes elapsed time. ○, in presence of 2 per cent glycerol, 2 minutes elapsed time.

these plots were, however, dependent on elapsed time as well as on initial spreading concentration. Thus for an elapsed time of 3 minutes the protein area of chymotrypsin was 1.40 sq.m. per mg. for an initial spreading concentration of 0.17 mg. per sq.m. and 0.95 sq.m. per mg. for an initial spreading concentration of 0.68 mg. per sq.m.

We have confined our measurements of trypsin to films spread on 5 per cent ammonium sulfate solution containing 2 per cent glycerol by volume. Trypsin films at all initial surface concentrations (0.18 mg. per sq.m. up to 0.72 mg. per sq.m.) and from elapsed times of 1 to 5 minutes showed a well defined break in the *FA versus F* plots which occurred at about 0.1

dyne per cm. Thus two linear segments were observed, one below 0.1 and another above 0.3 dyne per cm. The extrapolation of both linear segments to zero pressure yielded a film molecular weight of about 35,000. The slope of the *FA versus F* plot below 0.1 dyne per cm. corresponded to a protein area of about 2.5 sq.m. per mg., while the slope above 0.3 dyne per cm. corresponded to a protein area of 0.65 sq.m. per mg.

Rabbit serum γ-globulin yields a film molecular weight of about 170,000 when spread on 5 per cent ammonium sulfate solution containing 2 per cent by volume of glycerol. The initial surface concentration was 0.52 mg. per sq.m. and the elapsed time was 3 minutes. The protein area of this film was 0.91 sq.m. per mg. At lower initial surface concentrations, the film expanded greatly and its behavior on compression became erratic; so that no useful estimates of the film molecular weights could be made for films spread at lower initial surface concentrations. On 5 per cent ammonium sulfate in the absence of glycerol the *FA versus F* plot practically passed through the origin, indicating very extensive association.

Since the rabbit serum γ globulin contained a high concentration of antibody against egg albumin, interaction was attempted of spread films of antibody and of egg albumin on the surface of a 5 per cent ammonium sulfate to which 2 per cent by volume of glycerol had been added. No evidence of interaction could be discovered with a variety of relative film concentrations of antibody and of antigen.

Crystalline lysozyme from egg white supplied to us by Dr. Fraenkel-Conrat failed completely to spread on 5 per cent ammonium sulfate solution at pH 5.0 or on 5 per cent magnesium sulfate adjusted to pH 9.0 with sodium hydroxide.

DISCUSSION

In our previous publications from this laboratory we had reported linear plots for *FA versus F* for egg albumin, β-lactoglobulin, pepsin, and insulin (2, 3). It was, therefore, a source of considerable concern to us that the present, more extensive investigation has revealed that many protein films at least under certain circumstances do not yield linear *FA versus F* plots. In our previous measurements the film balance was not sensitive enough to make measurements below 0.1 dyne per cm. and, accordingly, relatively high film concentrations had to be employed. At these higher film concentrations, breaks in the *FA versus F* plots do not occur. Thus our previous measurements were entirely valid as far as they went.

We have considered a number of explanations for the departure of the *FA versus F* plots from linearity; *i.e.*, the departure from Equation 8.

It was considered possible that surface-active impurities were displaced from the surface as the film was compressed. A great deal of effort went into an attempt to eliminate these impurities, and while we were not com-

pletely successful, we did succeed in reducing them very greatly. This improvement in our technique had no influence on the *FA versus F* plots and, accordingly, we were forced to abandon this explanation.

It was thought possible that the break in the *FA versus F* plots was associated with some obscure wetting phenomenon of the Wilhelmy slides at low pressures. The float balance which does not involve wetting exhibited, however, identical breaks.

The breaks in the *FA versus F* plots could conceivably be due to a change in the film molecular weight as the film was compressed. An extrapolation of the *FA versus F* plot at pressures above the break point

FIG. 4.   FIG. 5.
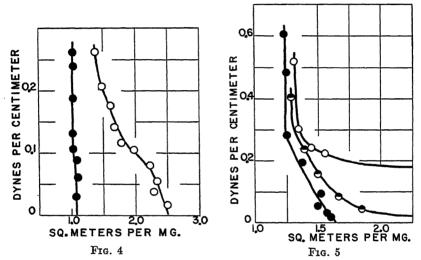
FIG. 4. Egg albumin area as a function of film pressure. 5 per cent ammonium sulfate, 5 minutes elapsed time. ○, 0.21 mg. per sq.m.; ●, 0.35 mg. per sq.m.

FIG. 5. Bovine serum albumin area as a function of film pressure. 5 per cent ammonium sulfate. 0.23 mg. per sq.m. initial spreading concentration. ○, 5 minutes elapsed time; ◐, 2 minutes elapsed time; ●, 1 minute elapsed time.

leads, however, to a smaller molecular weight than that at lower pressures. It does not appear probable that on compression the film molecular weight should decrease and, accordingly, this explanation was abandoned.

We now believe that the most reasonable explanation is that the protein molecules on the surface have a marked tendency to expand at low pressures, and as the surface is compressed and the film pressure increases, the individual molecules contract. The protein molecules can thus exist in two states; *i.e.*, the expanded State A and the compact State B with, no doubt, a number of intermediate forms. By assuming that Equation 8 is valid throughout the compression but that the protein area

(S_p) can change, it is possible to calculate the area occupied by the protein as a function of the film pressure. Shown in Fig. 4 are such curves for egg albumin at two initial spreading concentrations. Fig. 5 shows compression curves for bovine serum albumin at various elapsed times. Two experimental points have been omitted from this graph because of lack of space. For 5 minutes elapsed time the area is 4.0 sq.m. per mg. at 0.14 dyne per cm., and for 2 minutes elapsed time the area is 3.1 sq.m. per mg. at a film pressure of 0.03 dyne per cm. Shown in Fig. 6 are compression curves for insulin in the presence and in the absence of glycerol.

The factors which influence the protein area are (a) individuality of the protein; (b) increased elapsed time tends to increase the extent of expansion; (c) decreased initial spreading concentration tends to increase expan-
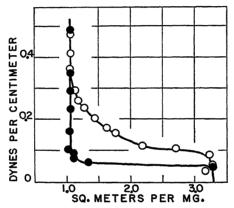
FIG. 6. Insulin area as a function of film pressure. 0.18 mg. per sq.m. initial spreading concentration. 5 per cent ammonium sulfate. O, in presence of 2 per cent glycerol, 2 minutes elapsed time. ●, in absence of glycerol, 5 minutes elapsed time.

sion; (d) glycerol tends to increase the protein area. There are no doubt other factors which influence the protein area, such as temperature, presence of heavy metals, and hydrogen and hydroxyl ions.

We have in this paper attempted to evaluate the spread film technique for the determination of the molecular weight of proteins. By and large, the results are encouraging provided not too high an accuracy is demanded. It is clear that there exists a simple correspondence between the film molecular weights of proteins and the molecular weights of these proteins as determined by osmotic pressure and by the ultracentrifuge. The intercept of the *FA versus F* plots will yield an unambiguous molecular weight. The difficulty arises in making such an extrapolation. At low pressures which are most important for making the correct extrapolation, the protein molecules may undergo expansion and, accordingly, the *FA versus F*

plot may not be linear. We are not hopeful of perfecting a reliable and convenient surface balance which will give accurate film pressures much below 0.01 dyne per cm. Surface-active impurities and other disturbing factors become very important at these low pressures. We do feel, however, that if a protein film is studied at various initial spreading concentrations and for various elapsed times the film molecular weight of a given protein can be estimated within ± 10 per cent, which would represent the extreme variation to be expected. It has been our experience that the float type of surface balance is more convenient to use than the Wilhelmy balance, at least in the low pressure region.

SUMMARY

1. An equation of state for spread films of protein in the low pressure region has been derived.

2. Pressure-area measurements on films of egg albumin, bovine serum albumin, rabbit γ-globulin, insulin, pepsin, chymotrypsinogen, chymotrypsin, and trypsin have been made in the low pressure region and under a variety of conditions.

3. It has been found that films of egg albumin, bovine serum albumin, insulin, and trypsin under certain conditions do not give linear FA versus F plots.

4. It has been suggested that the protein molecules can exist on the surface in an expanded state (State A) and in a compact state (State B). The area occupied by the protein molecules on the surface has been calculated as a function of the film pressure for several conditions.

5. It has been found that under certain circumstances films of pepsin, chymotrypsinogen, chymotrypsin, and γ-globulin associate on the surface.

6. The film molecular weights calculated for the proteins studied are in good agreement with the molecular weights of these proteins in solution.

7. Lysozyme does not form surface films under the conditions employed.

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THE RÔLE OF GLUTAMIC ACID IN ARGININE SYNTHESIS BY LACTOBACILLUS ARABINOSUS*

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Stokes and Gunness (1) and Lyman *et al.* (2) have found that the amino acid requirements of *Lactobacillus arabinosus* 17-5 vary with the composition of the basal medium. It was observed (2) that the organism does not require arginine, phenylalanine, and tyrosine if grown in the presence of pyridoxamine and carbon dioxide. To elucidate the manner in which carbon dioxide is utilized in the chemical reactions leading to the synthesis of these amino acids, information was sought regarding other specific metabolites that might be involved. It was found that the rapid growth of the organism in the absence of arginine required increased amounts of glutamic acid. The experiments reported here were designed to give further information concerning the relationship of glutamic acid to arginine synthesis in *L. arabinosus*.

EXPERIMENTAL

The effect of specific nutrients and metabolic inhibitors on the growth of *L. arabinosus* was studied under conditions of growth that required the organism to synthesize its own arginine.

The cultures were grown in culture medium placed in sealed flasks containing a gas phase of 6 per cent carbon dioxide in air as previously described (2).

In the tests in which glutamine and carbamyl-L-glutamic acid were used, these compounds were sterilized through filtration in sintered glass bacterial filters and added aseptically. The technique used for handling the organisms and inoculating the culture medium was the same as that described for the microbiological determination of amino acids (3). The composition of the medium was the same as previously described (2) except that pyridoxamine was substituted for pyridoxine in all cases.

The amount of growth occurring in the culture tubes was measured in either of two ways. In some cases the acid produced was titrated with 0.1 N sodium hydroxide. In other cases, turbidimetric measurements were

made with the Klett-Summerson photoelectric colorimeter and a No. 66 light filter. Culture medium which had not been inoculated was used as a blank.

Carbamyl-L-glutamic acid was prepared by the method of Nyc and Mitchell (4) and methionine sulfoxide by the method of Roper and McIlwain (5).
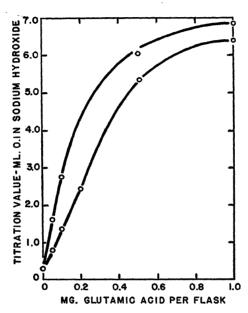
FIG. 1. Glutamic acid requirements for the growth of *L. arabinosus* in media with and without arginine. The titration values are for 5 ml. aliquots from 10 ml. cultures. 4 γ of pyridoxamine and 2 γ of glutamine per flask. Gas phase, 6 per cent CO_2 in air.

RESULTS AND DISCUSSION

Fig. 1 shows the increased requirement of *L. arabinosus* for glutamic acid in medium not containing arginine but in the presence of carbon dioxide and pyridoxamine. These data indicate that glutamic acid probably functions in arginine synthesis. Many chemical reactions involving glutamic acid have been shown in the literature to take place in living cells. Three of these reactions which might be related to arginine synthesis in lactic acid bacteria are as follows: (1) Glutamic acid might function by supplying the imino group to citrulline through the transimination reaction first discussed by Borsook and Dubnoff (6). (2) Glutamic acid might form a part of a mechanism for the fixation of carbon dioxide through the intermediary

formation of carbamyl-L-glutamic or carbamino-L-glutamic acid as discussed by Cohen and Grisolia (7, 8). (3) Glutamic acid might function through the intermediary formation of glutamine, which in turn might function in a number of different ways. The presence of proline in the basal medium eliminates a possibility not mentioned above that glutamic acid is functioning in the system, glutamic acid → proline → ornithine.

With regard to the first of the possibilities listed above, it was found that the addition of citrulline to an arginine-free medium removed the requirement for additional glutamic acid. This finding indicates that the function of the increased glutamic acid concentration is not associated with the transiminase reaction. Ratner and Pappas (9) found that in animal tissues aspartic and not glutamic acid functions in the transimination of citrulline to give arginine.

To test the second reaction, organisms were grown in an arginineless medium to which 2 mg. of carbamyl-L-glutamic acid (C-L-G) were added per flask. Fig. 2 shows that the added C-L-G can only partially remove the additional requirements for glutamic acid in an arginineless medium. These data do not indicate that C-L-G is non-functional in the fixation of carbon dioxide to ornithine, but do indicate that this function does not impose sufficient requirements on glutamic acid to account for the differences indicated in Fig. 1. It is worthy of note that C-L-G is unable to replace the growth requirements for glutamic acid in this organism.

The possibility that glutamic acid functions in arginine synthesis in this organism through the intermediary synthesis of glutamine was tested by the use of α-amino-γ-(methylsulfinyl)-butyric acid, better known as methionine sulfoxide (MSO). This compound has been shown to be an effective inhibitor in the formation of glutamine by Borek et al. (10), and others (11–13).

Fig. 3 shows that the addition of 60 mg. of MSO per flask (10 ml. volume) completely blocked the growth of the organism in the absence of arginine and that glutamine removed the inhibition produced by MSO. In these experiments each tube contained 1 mg. of L-glutamic acid. Other experiments showed that the inhibition could be removed by adding L-glutamic acid as well as by adding glutamine. The top curve, representing growth with no inhibitor, shows only small effects due to the addition of increasing amounts of glutamine, while the bottom curve, representing growth with inhibitor, is almost a direct function of added glutamine. These curves imply that glutamine is required for arginine synthesis and, therefore, must be either supplied in the medium or synthesized from glutamic acid.

In order to show conclusively that glutamine is required for arginine synthesis and that the inhibitory action of MSO in preventing the synthesis

of glutamine is the specific factor which limits arginine synthesis, it was desirable to provide a complete medium in which maximum growth could be obtained in the presence of the inhibitor. This was accomplished by the addition of 2 mg. of L-asparagine and 10 mg. of ammonium chloride to each 10 ml. of medium. Fig. 4 represents the growth of organisms grown in medium containing the above supplementation.

FIG. 2.

FIG. 3.
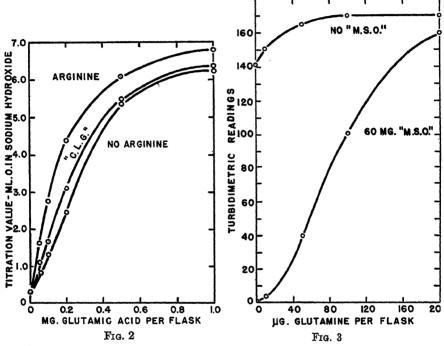
FIG. 2. Effect of carbamyl-L-glutamic acid on the glutamic acid requirements of *L. arabinosus* in media without arginine. 2 mg. of carbamyl-L-glutamic acid per flask. Other conditions as in Fig. 1.

FIG. 3. Effect of methionine sulfoxide (MSO) and glutamine on the growth of *L. arabinosus* in a medium without arginine. Growth period 24 hours, 34°, 6 per cent CO_2 in all flasks. A turbidimetric reading of 0 indicates no growth.

From these data it is concluded that the requirement of glutamine for growth in this supplemented medium is very slight, that MSO is not inhibitory to rapid growth when arginine is present, and that glutamine is required for arginine synthesis.

Four possible mechanisms by which glutamine might function in the synthesis of arginine by *L. arabinosus* are as follows: (1) Glutamine might be converted to ornithine by reduction. (2) It might function by supply-

ing nitrogen for the formation of carbamyl-L-glutamic acid from carbamino-L-glutamic acid and serve in a mechanism for carbon dioxide utilization. (3) It might function in fixing ammonia to δ-carbamino-L-ornithine with the resulting formation of δ-carbamyl-L-ornithine or citrulline.   (4) It may

FIG. 4.

FIG. 5.
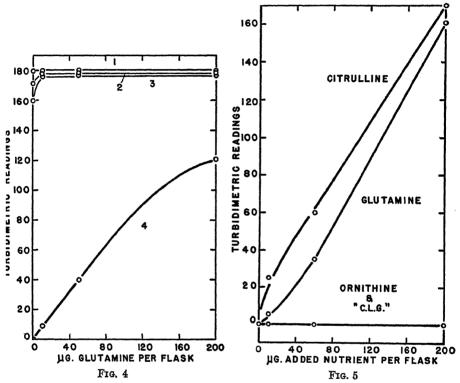
FIG. 4. Effect of methionine sulfoxide and glutamine on the growth of *L. arabinosus* in a medium supplemented with 2 mg. of L-asparagine and 10 mg. of ammonium chloride per flask.   Curve 1, 2 mg. of arginine per tube; Curve 2, no arginine; Curve 3, 2 mg. of arginine plus 60 mg. of MSO; Curve 4, no arginine, 60 mg. of MSO.   All flasks, 6 per cent CO_2.

FIG. 5. Effect of citrulline, glutamine, ornithine, and carbamyl-L-glutamic acid on the growth of *L. arabinosus* in arginine-free medium.   60 mg. of methionine sulfoxide; 2 mg. of L-asparagine; 10 mg. of ammonium chloride and 6 per cent CO_2 in all flasks.

function in a more direct manner, either yet undescribed or possibly related to the system described by Leuthardt for urea formation (14, 15).

To evaluate the first and second possibilities an experiment was carried out in which ornithine and carbamyl-L-glutamic acid were substituted for glutamine in an arginineless medium.   As is shown in Fig. 5, neither of

these compounds could replace glutamine for the growth of *L. arabinosus*. It is then apparent that the key function of glutamine is not as an intermediate in ornithine synthesis or in supplying ammonia to carbamino-L-glutamic acid.

The third possibility with respect to the function of glutamine in arginine synthesis appears to be the most probable at the present time. This interpretation is in line with the data given in Fig. 5 which shows that citrulline is more effective than glutamine in promoting growth in an arginineless medium. Experiments designed to give more positive evidence with respect to both the third and fourth possibilities are in progress.

SUMMARY

1. The glutamic acid requirements for the rapid growth of *Lactobacillus arabinosus* 17-5 were found to be increased when arginine was omitted from the medium and carbon dioxide and pyridoxamine were supplied.

2. In the absence of arginine the growth of the organism was blocked by the addition of methionine sulfoxide. This inhibition was removed by adding glutamine.

3. Neither carbamyl-L-glutamic acid nor ornithine would promote growth in the absence of arginine and glutamine when the medium contained methionine sulfoxide.

4. Citrulline was more effective than glutamine in replacing arginine in a medium containing methionine sulfoxide, indicating that the function of glutamine may be to supply ammonia for the synthesis of citrulline from δ-carbamino-L-ornithine.

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ELECTRON MICROSCOPY OF CRYSTALLINE EDESTIN

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Existing electron microscopes, when properly adjusted, are capable of resolutions of about 20 A or better, thus permitting the direct observation of spherical protein particles having molecular weights of about 4000. However, adequate instrumental resolution alone does not insure that the information recorded will be useful; the images of randomly disposed minute particles which are commonly observed in protein preparations are likely to be of dubious value unless they can be identified and characterized. The value and reliability of electron microscope observations are much increased if molecules can be observed in crystalline configurations in which they produce identifiable and accurately measurable patterns. Although the macromolecular units of virus crystals have been successfully observed by Wyckoff (1) and others, none of the non-virus proteins of smaller molecular dimensions has hitherto been studied in the crystalline state by electron microscopy. Edestin crystals were chosen for the initial experiments because of their relatively high stability and because direct quantitative evidence concerning the dimensions of the edestin molecule is of interest, as it bears on numerous previous experiments on the same protein by indirect methods.

EXPERIMENTAL

Methods—Crystalline edestin was extracted from hemp seed according to the method described by Bailey (2) and recrystallized up to six times. Specimens for electron microscopy were prepared by dissolving the crystalline protein in 2.5 per cent NaCl at 55° and applying 1 drop of the warm solution to a conventional electron microscope specimen screen with collodion film. Since the solubility of edestin decreases rapidly under about 35°, crystals form in a few minutes on the supporting film as the drop cools. In order to remove salt and uncrystallized protein, the specimen was flushed quickly with a stream of water from a medicine dropper, washed with 75 per cent alcohol, and dried.

Crystalline globulin from squash seed (Hubbard squash) was prepared according to a method described by Vickery *et al.* (3). Crystals for electron microscopy were obtained at room temperature by adding water slowly to a solution of the protein in 10 per cent NaCl until a visible precipitate formed. 1 drop of the crystallizing suspension was then applied to a conventional specimen mount, washed, and dried.

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In preliminary experiments, specimens were shadowed and observed directly, but electron scattering by the underlying protein was too great to permit satisfactory observation of the metallized surface structures. The difficulty was overcome by evaporating a thin film of SiO at normal incidence over the shadowed surface and subsequently removing the collodion film with acetone and the protein by immersing the specimen in 0.05 N HCl for 1 or 2 minutes. The metal always adheres to the SiO film when the underlying material is removed. Although the original collodion film is between the SiO and the metal grid, the transfer film is never loosened from its support, probably because acetone does not penetrate much beyond the open areas. Metals such as U, Ni, Cr, and Pt were used for shadowing, usually with a shadow to height ratio of 3:1 and in calculated weights of about 2.5×10^{-6} gm. per sq. cm. or less in the plane of the specimen. SiO was applied in weights of between 1.0 and 1.5×10^{-6} gm. per sq. cm. The rigidity of SiO and the absence of manipulative procedures on unsupported thin films are deemed advantages of the method. It is noteworthy that the method may also be used for the examination of frozen aqueous substances (4), which suggests the possibility of examining protein crystal surfaces without removing water of crystallization.

Electron Microscope Results—Edestin crystals, grown on a collodion film in this manner, appear most commonly as equilateral triangles with truncated corners, as shown in Fig. 1, although occasionally twinned forms and crystals with hexagonal outline occur. Significant portions showing molecular nets on the crystals of Fig. 1 have been circumscribed and reproduced at higher magnification below the micrograph. Although there is considerable disorder in parts of the triangular faces, due either to distortion produced by drying or to non-crystalline surface deposits, areas are always visible showing well defined hexagonal nets with rows of adjacent molecules oriented parallel to the triangle edges as shown in Figs. 1, *a* and *c*. On sloping faces at truncated corners the net is rectangular, as shown in the enlarged portion, Fig. 1, *b*. So far as can be determined from the heights of steps as estimated from shadow lengths and from examination of individual particles, the molecules appear to be approximately spherical, about 80 A in diameter. From a study of these features, it has been concluded, as has been previously reported in brief (5), that the observed structures closely approximate orthographic projections of a face-centered cubic lattice, with the triangular faces representing (111) planes and the rectangular nets at the truncated corners representing contiguous (100) planes. A photograph of a model of a crystal as deduced from the electron microscope observations is shown in Fig. 2.

On the assumption that the lattice is face-centered cubic, it is possible to calculate the dimensions of the unit cell from measurements on the
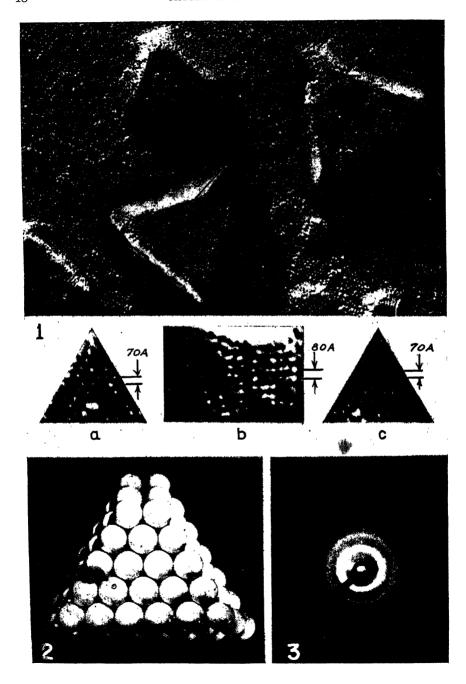
(111) face alone. The perpendicular distance between rows of molecules may be obtained with fair accuracy by measuring the distance across several adjacent rows and dividing by the total number. In over thirty such measurements from different crystals the distance varied between 68 and 72 A, with an average of 69.7 A. It is readily calculated from this that $a_o = 114$ A. The distance between the prominent rows in the (100) face, as shown in the rectangular inset in Fig. 1, is about 80 A, which is, of course, consistent. According to the simplified model shown in Fig. 2, the diameter of the molecules in the dry state is close to 80 A, but perfect sphericity in the actual crystals is unlikely.

From the dimension of the unit cell, together with a value for the crystal density, the dry molecular weight of edestin may be calculated. Published values for the density of dry edestin crystals vary between about 1.30 and 1.35 (6), according to the methods used, yielding values for the molecular weight of 290,000 and 300,000, respectively, which are in excellent agreement with the value of 310,000 reported by Svedberg and Pedersen (7) from ultracentrifugal data.

An interesting feature, apparent in Fig. 1, is the tendency for a monolayer to grow out over the background from triangular crystal edges. The structure is two-dimensional, approximating hexagonal packing close to the crystal edge and tending gradually toward a rectangular pattern away from the crystal. The general background is mostly non-crystalline although occasionally small areas exhibit hexagonal patterns, evidently where crystals are beginning to form.

The crystal structure of the similar globulin from squash seed (3) is identical with that described for edestin, as far as the electron microscope can show. The perpendicular distance between rows of molecules on the (111) faces varied from 62 to 79 A, with an average of 69.4 A.

X-Ray Results—X-ray powder patterns of edestin crystals from the same preparation used for electron microscopy were made by Dr. R. S. Bear using a small angle camera. Crystals were dispersed in 2.5 per cent NaCl, sealed in a glass capillary about 1 mm. in diameter with a 25 μ wall, and exposed to copper radiation ($\lambda = 1.54$ A) through a nickel filter. A magnified reproduction of the inner part of a pattern taken at 9.6 cm. from the specimen is shown in Fig. 3. The first three relatively intense rings are visible in the reproduction and several others, mostly of lower intensity, have been observed at wider angles on the original films. Results of measurements on the first four rings are listed in Table I, where the final column shows the calculated value of a_o for each of the rings, on the assumption that the lattice is cubic and that the appropriate indices are those listed in the fourth column. Values for a_o are consistent within limits of experimental error and, since sets of indices are all odd or all

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a   b   c

70A   80A   70A

2   3

even, the results suggest a face-centered cubic lattice. The average value for a_o is 140 A. Other rings, not listed, are too faint or broad to allow unequivocal assignment of indices, but in all instances, indices consistent with the above results may be assigned within experimental error.

The difference between the x-ray and electron microscope values for the unit cell dimensions is attributed to hydration. The water content of wet edestin crystals has been measured by Bailey (2), whose results show that wet crystals contain about 39 per cent water by weight. If the density of the dry protein is assumed to be 1.35 and the cell dimension for the perfectly dry crystals is 114 A, it is readily calculated that this quantity of water would increase the dimension to 140 A. In the preparative procedure for electron microscopy, the crystals were placed in a vacuum and probably heated slightly by radiant heat from the evaporation filaments. There is no assurance that the crystals were completely dehydrated, but the close correspondence between the wet cell dimension

TABLE I

X-Ray Powder Pattern of Wet Edestin Crystals

Intensity	$\dfrac{2 \sin \theta}{\lambda}$	$h^2 + k^2 + l^2$	(hkl)	$a_o = \dfrac{\lambda}{2 \sin \theta} \sqrt{h^2 + k^2 + l^2}$
				A
Very strong.........	0.0124	3	(111)	140
Strong.............	0.0201	8	(220)	141
Weak..............	0.0310	19	(331)	141
Very weak.........	0.0406	32	(440)	139

calculated from electron microscope data and that determined directly by x-ray diffraction indicates that the value of 114 A applies to the protein in a state of thorough dehydration.

DISCUSSION

Although the molecular weight of edestin calculated from electron microscopy agrees with that determined from ultracentrifugal data, the conclusion that the edestin molecule is approximately spherical is at variance with deductions regarding its shape based on measurements of

FIG. 1. Electron micrograph showing surface structure of edestin crystals shadowed with nickel. $\times$ 100,000. Circumscribed areas *a*, *b*, and *c* are shown below the micrograph at a magnification of $\times$ 300,000.

FIG. 2. Model of edestin crystal with a face-centered cubic lattice, exposing a (111) face.

FIG. 3. Central portion of 9.6 cm. x-ray powder pattern of wet edestin crystals, enlarged 3 times. The inner ring corresponds to an 81 A spacing.

the dielectric dispersion of edestin solutions. Oncley (8) has reported that such data indicate the molecule has an axial ratio of 9:1 when dissolved in 2 M glycine. Also, frictional ratios obtained from sedimentation experiments may be interpreted as evidence of an asymmetry ratio as high as 4:1, depending on assumptions concerning hydration of the molecule (6). However, if a rather high degree of hydration is assumed for the molecule in solution, these latter data would be consistent with a nearly spherical molecule, in agreement with the electron microscope data. Electron microscope observations, either on edestin crystals or on the non-crystalline background material, are incompatible with a high degree of asymmetry. Conceivably the shape is different in solution, but such an explanation would not be acceptable without independent proof. It would be of considerable interest to establish the reason for the apparent discrepancy between the methods.

The conclusions concerning the structure of edestin crystals derived from electron micrographs are strengthened by the agreement with available x-ray data, when allowance is made for differences in hydration. It is to be noted, however, that the x-ray data are not so extensive or precise that they provide a unique interpretation by themselves. Since it is often the case that x-ray patterns of proteins are not well developed, the value of complementary evidence from electron microscopy in this type of investigation is apparent.

No previous small angle x-ray results have been reported on crystalline edestin. Crowfoot and Fankuchen (9) obtained powder patterns from air-dried crystals of a similar globulin from tobacco seed and concluded that the observed diffractions are consistent with a face-centered cubic lattice having $a_o = 123$ A. In a later review, however, Fankuchen (10) expressed some dissatisfaction with the quality of the x-ray patterns on which the conclusions were based. Since the results on tobacco seed globulin as reported are close to those found for edestin and squash seed globulin, it seems probable that the three are similar in structure, but additional electron microscope or x-ray data on tobacco seed globulin would be desirable.

It is of interest to add that, following this initial investigation, the methods described have been applied to a number of other crystalline proteins. Electron micrographs have been obtained showing regular and extensive molecular patterns on the faces of crystalline canavalin, concanavalin B, and catalase, to cite examples which will be described later. The results indicate that the electron microscope is capable of providing precise information regarding the size and shape of molecules in protein crystals.

SUMMARY

1. Electron micrographs are described which show the molecular configuration at the surface of edestin crystals. It is concluded that the lattice is face-centered cubic with $a_o = 114$ A in the dry state.

2. The edestin molecule appears as a particle about 80 A in diameter. With an assumed density of 1.35, the molecular weight is calculated as 300,000 in the dry state, in good agreement with values deduced from ultracentrifugal data.

3. Measurements from x-ray powder patterns of wet crystals are presented, indicating a face-centered cubic lattice having $a_o = 140$ A, which larger value is consistent with published data concerning the water content of hydrated edestin crystals.

4. As far as the electron micrographs can show, the structure of crystals of squash seed globulin (Hubbard squash) is identical with that of edestin.

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OXIDATION IN VIVO OF THE METHYL GROUPS OF CHOLINE, BETAINE, DIMETHYLTHETIN, AND DIMETHYL-β-PROPIOTHETIN*

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It has recently been demonstrated in this laboratory (1–3) that "biologically labile" methyl groups can be oxidized to CO_2 in the rat. This has been established by feeding methionine containing radioactive carbon in the methyl group and demonstrating the presence of radioactive CO_2 in the expired air. In the present communication a comparison is made of the rates of oxidation of methyl groups administered as choline, betaine, dimethylthetin (4), and dimethyl-β-propiothetin (5). These compounds, labeled with C^{14} in one methyl group, have been prepared and injected intraperitoneally into rats. The radioactivity of the CO_2 in the expired air was measured at intervals during a 24 hour period.

EXPERIMENTAL

Synthesis of Labeled Compounds[1]—For the synthesis of choline, methyl iodide prepared from 3.88 mM of methanol containing 1.15 mc. of C^{14} was distilled in a stream of dry nitrogen into a solution of 346 mg. (3.9 mM) of dimethylaminoethanol in 5 ml. of ethanol, contained in a scrubber cooled in a solid CO_2 bath at −60°. After the distillation was complete, the cooling bath was removed and the mixture was allowed to stand at room temperature for 17 hours. The choline iodide was collected, the filtrate was evaporated to dryness, and the two fractions were recrystallized separately from boiling absolute ethanol. The two samples were then combined, dissolved in approximately 40 ml. of absolute ethanol, and shaken with a slight excess of AgCl. The AgI was removed by filtration and washed with ethanol and then with hot water. After evaporation of the filtrate and

[1] The starting material for all the preparations was radioactive methanol purchased from the Clinton Laboratories of the Monsanto Chemical Company on allocation from the United States Atomic Energy Commission; the values for the radioactivity are taken from data supplied by the company. The methanol was converted to methyl iodide by the method of Melville, Rachele, and Keller (6).

washings to dryness, the residue was recrystallized from ethanol-ether. The yield of choline chloride was 52 per cent of the theoretical amount on the basis of the methanol. A small sample was converted to the chloroplatinate for analysis.

$C_{10}H_{28}O_2N_2 \cdot PtCl_6$. Calculated, Pt 31.66; found, Pt 31.72

The radioactivity was determined after combustion of a small sample of the chloroplatinate and conversion of the CO_2 to barium carbonate, according to the method described previously (7). The specific activities of the other labeled methyl compounds reported in this communication were also determined after oxidation according to this procedure. From the activity of the chloroplatinate, the specific activity of the choline chloride was calculated to be 3.82×10^5 c.p.m. per mg.

For the preparation of betaine, methyl iodide made from 3.91 mM of methanol containing 1.23 mc. of C^{14} was distilled in a stream of nitrogen into a solution of 520 mg. (4.2 mM) of dimethylglycine sodium salt in 8 ml. of 75 per cent ethanol, contained in a scrubber cooled to $-8°$ to $-10°$. When the distillation was complete, the inlet tube was removed and the reaction vessel was tightly stoppered and heated in an oil bath at 70° for 85 minutes. The mixture was then evaporated *in vacuo* to a small volume. 125 ml. of a saturated aqueous solution of ammonium reineckate were added and the mixture was acidified with HCl, cooled in ice overnight, and filtered. The precipitate was dissolved in 40 ml. of 0.1 N NH₄OH and shaken with excess silver oxide until all the color had been removed. The precipitate of silver reineckate was collected and washed with water; the filtrate and washings were combined, heated to 60°, and aerated for 4 hours to remove the ammonia. The solution was then concentrated to a volume of about 20 ml., acidified with HCl, filtered, and evaporated to dryness. The residue was dissolved in the minimum amount of boiling ethanol, filtered, and cooled in ice. The first crop of crystals was collected; the filtrate was evaporated to a volume of 3 ml. and a second crop was obtained by cooling; the two crops were combined. After this material was dissolved in ethanol and reprecipitated with ether several times, the betaine hydrochloride, obtained in a yield of 30 per cent of the theoretical amount on the basis of the methanol, had a specific activity of 3.30×10^5 c.p.m. per mg.

$C_5H_{12}O_2ClN$. Calculated, Cl 23.08; found, Cl 23.02

For the preparation of dimethyl-β-propiothetin, methyl iodide prepared from 4.11 mM of methanol containing 1.23 mc. of C^{14} was distilled in a stream of dry nitrogen into a mixture of 525 mg. (4.37 mM) of β-methyl-mercaptopropionic acid, 3 ml. of formic acid, and 0.3 ml. of water, con-

tained in a U-shaped receiver which was cooled in a solid CO_2 bath. The receiver was then stoppered securely and allowed to stand at room temperature for 64 hours. The reaction mixture was dissolved in water and shaken with 800 mg. of AgCl. The AgI was collected and washed well with hot water. After evaporation of the filtrate and washings to dryness the residue was recrystallized three times by dissolving in boiling ethanol and precipitating with dry ether. The dimethyl-β-propiothetin hydrochloride weighed 512 mg. (73 per cent of the theoretical amount on the basis of the methanol) and had a specific activity of 2.77×10^5 c.p.m. per mg.

$C_5H_{11}O_2ClS$. Calculated, Cl 20.78; found, Cl 20.63

The procedure employed for the synthesis of the dimethylthetin was exactly the same as that described for dimethyl-β-propiothetin. The starting materials in this case were methyl iodide, prepared from 4.13 mM of methanol containing 1.26 mc. of C^{14}, and S-methylthioglycolic acid (465 mg., 4.37 mM). The dimethylthetin hydrochloride obtained after three recrystallizations from ethanol-ether weighed 374 mg. (58 per cent of the theoretical amount on the basis of the methanol) and had a specific activity of 2.90×10^5 c.p.m. per mg.

$C_4H_9O_2ClS$. Calculated, Cl 22.64; found, Cl 22.36

Oxidation Experiments in Vivo—Four male rats of the Rockland strain (Rats 1 to 4) weighing 100 to 120 gm. were placed on an amino acid diet identical with one described previously (Diet II (8)) except that the level of DL-methionine was 0.6 per cent. The animals were maintained on this diet, which was choline-free and contained 0.4 per cent of cystine, until they had reached a weight of 140 ± 4 gm. Aqueous solutions (0.7 ml.) of the labeled methyl compounds were then injected intraperitoneally at approximately the same time of day in each case (9.30 to 11 a.m.). The compounds were administered in the following amounts, providing equivalent amounts of methyl groups (0.21 to 0.22 mM CH_3—): choline chloride 10.0 mg. (Rat 1), betaine hydrochloride 10.9 mg. (Rat 2), dimethylthetin hydrochloride 16.7 mg. (Rat 3), and dimethyl-β-propiothetin hydrochloride[2] 18.0 mg. (Rat 4). The animals were placed immediately in the open circuit metabolism apparatus described previously (3) and allowed free access to food. The food consumption over the 24 hour period was as follows: 11 gm. (Rat 1), 9 gm. (Rat 2), 8 gm. (Rat 3), and 12 gm. (Rat 4).

The expired CO_2 was collected continuously in two absorbers, each containing 400 ml. of 2.5 N NaOH, which were changed every hour for the first 7 or 8 hours and again 24 hours after administration of the labeled

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[2] In this case the administered compound was a 59:41 per cent mixture of labeled and ordinary dimethyl-β-propiothetin hydrochloride.

compounds. Aliquots of these solutions were converted to $BaCO_3$ for determination of the radioactivity. The precipitation and counting procedures were identical with those previously described (3, 7).

The per cent of the injected radioactivity which was expired as CO_2 during various time intervals after administration of the C^{14}-labeled choline, betaine, dimethylthetin, and dimethyl-β-propiothetin was determined. The results are presented in Fig. 1. These experiments were repeated on another set of animals with results which corresponded closely for each compound with those shown in Fig. 1.
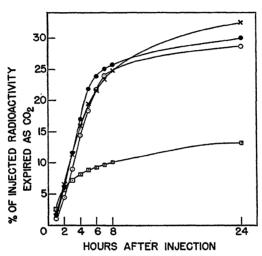
FIG. 1. Per cent of the radioactivity which was expired as CO_2 at given time intervals after injection of choline ($\square$), betaine ($\times$), dimethylthetin ($\bullet$), and dimethyl-β-propiothetin ($\bigcirc$), labeled with C^{14} in one methyl group. Each point on the curves represents the summation of the percentages of the injected radioactivity expired up to the time indicated along the abscissa.

DISCUSSION

It will be noted that, when betaine, dimethylthetin, dimethyl-β-propiothetin, and choline are administered intraperitoneally, at levels providing equivalent amounts of methyl groups, the percentage of methyl groups oxidized is greater in the case of the first three compounds than with the choline. The highest level of radioactivity in the expired CO_2 occurred some time during the first 4 hours after administration of the compound in all cases.

The fact that the methyl groups supplied in the form of choline are converted to CO_2 more slowly than the methyl groups administered as betaine or the two sulfonium compounds may be due to the dilution of the radioactive choline by body choline or may be simply a reflection of the rapid

diversion of the choline into reactions other than those involving oxidation of the methyl groups. Nevertheless, the data would seem to indicate that oxidation of the methyl groups of betaine, dimethylthetin, and dimethyl-β-propiothetin does not require that these groups be first transferred to choline.

It is known, for rats on a labile methyl-free diet containing homocystine, that dimethylthetin and dimethyl-β-propiothetin have growth-promoting activity which closely approximates that of choline and betaine (4, 5, 8). The thetins are also active methyl donors for the methylation *in vitro* of homocysteine by liver and kidney homogenates (9). The close similarity between the rates of oxidation of the methyl groups of betaine and the two thetins provides another example of the rather remarkable metabolic similarity between betaine and its sulfur analogues.

SUMMARY

The synthesis of choline, betaine, dimethylthetin, and dimethyl-β-propiothetin labeled with C^{14} in one methyl group has been described. The percentage of the methyl groups of these compounds which was expired as CO_2 by the rat over a period of 24 hours was measured. The methyl groups administered in the form of betaine, dimethylthetin, and dimethyl-β-propiothetin were found to be oxidized to a greater extent than the methyl groups administered as choline. A high degree of similarity was observed in the rates of oxidation of the methyl groups of betaine and the two thetins.

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ON THE ORIGIN OF CERTAIN SERUM PEPTIDASES AS INDICATED BY EXPERIMENTAL HEMOLYTIC ANEMIA IN DOGS*

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Since the discovery that simple peptides are hydrolyzed by human and animal sera (1–5), efforts have been made to study the quantitative variation in these peptidases with the hope that they could be correlated with other metabolic activities. Indeed, it has been demonstrated by a number of investigators that increased serum peptidase levels are found in fever, shock, burns, etc. (1, 6, 7).

It is likely that the enzymes present in serum are derived from the secretion or dissolution of various cellular elements. Fruton (8, 9) has suggested that the serum peptidases might originate from the breakdown of lymphocytes. In support of this idea, attention was drawn to the high concentration of such enzymes in lymphoid tissue, and in leucocytes and lymphocytes (9–11), and to the fact that the abnormal conditions, such as shock, burn, fever, etc., which produce peptidase increases are also known to augment adrenal cortical secretion by way of pituitary stimulation; this hormonal system has been shown to cause breakdown of tissue and circulating lymphocytes (12). Experimental support of this concept is derived from the observation that injection of pituitary adrenocorticotropic hormone or of cortical steroids led to an increase in the level of the serum peptidases of mice (13). In view of the wide-spread distribution of the peptidases in animal tissues, Fruton has also mentioned the possibility that these enzymes may not be present in the characteristic cells of various tissues but may possess a common origin in the lymphocytes or leucocytes present in the tissues.

A previous study from this laboratory (14) has already shown that although the injection of adrenotropic hormone into two normal, male, human subjects produced a marked lymphopenia there was little or no increase in the level of serum peptidases. A study were therefore undertaken to determine whether these peptidases could also originate from other cells. It was found that anemia in dogs produced by the adminis-

tration of phenylhydrazine was associated with a marked rise of serum peptidases concurrent with the breakdown of erythrocytes. Moreover, injection of cortical steroids into the same dogs, following their complete recovery from the anemia, produced a marked lymphopenia but had no effect on the serum peptidase level. These experiments suggest that, in the dog, serum peptidases probably originate in large part from the dissolution of erythrocytes. Indeed, peptidases have been directly demonstrated by others to be present in mammalian erythrocytes (6, 15) and our own preliminary findings confirm this. This suggests that peptidases do occur in a variety of mammalian cells. This is certainly consistent with the broad distribution of similar enzymes in invertebrate tissues, bacteria, fungi, and green plants (16).

The high levels of serum peptidases found after erythrocyte breakdown indicate that the usual methods of studying the level of serum peptidases must be evaluated with great caution. Obviously, any handling of blood which produces even slight hemolysis may cause an increase in peptidase levels. It may be that, in some instances, it is the dissolution of the red cells which is the determining factor in the serum peptidase levels. Zamecnik, Stephenson, and Cope (6) have previously indicated that part of the increase in peptidases in post-burn sera should be ascribed to hemolysis. Since the first report of our work (17), Schwartz and Engel (18) have independently found that administration of adrenal cortical extracts in mice and rats does not produce a significant alteration of serum peptidase levels, and have called attention to the possible liberation of peptidases by hemolysis. Likewise, Johansen and Thygersen (15) have pointed out that erythrocytes, which contain 40 to 50 times more peptidases than serum, may be regarded as the main source of the serum peptidases. Nevertheless, it should be made clear that the erythrocytes do not necessarily represent the only source of the serum peptidases. The high levels of peptidases found in leucocytes and in lymphoid tissue, such as thymus (9), make it quite likely that these could also contribute peptidases to serum.

In this paper, we shall describe our experimental findings on dogs, and some of our data on normal and abnormal human sera will be presented.

<div align="center">EXPERIMENTAL</div>

Since many peptides are hydrolyzed by serum, the selection of substrates must be made in terms of particular enzymes and our knowledge of their specificity. While this choice is somewhat arbitrary, we have attempted to avoid the use of compounds which are split by more than one enzyme, since this would render interpretations difficult or uncertain. Serum contains a polypeptidase, which is probably an aminotripeptidase similar to that obtained from animal tissues (9, 19). The usual substrate

employed for the study of this enzyme in serum has been L-leucylglycyl-glycine. However, since leucine aminopeptidase also acts on the leucine tripeptide, we have preferred to use diglycylglycine in order to avoid the possible complication caused by a dual action on the same substrate. For leucine aminopeptidase, we have used L-leucinamide as the substrate in preference to the more commonly employed L-leucylglycine which may be attacked by other enzymes (20). The unique enzyme prolidase, which attacks imino peptide bonds, is found in serum in large quantity (20); this peptidase has been studied by its action on glycyl-L-proline. Gly-cylglycine was used as the substrate for the cobalt-activated enzyme, glycylglycine dipeptidase (21).

The experiments were performed with serum separated from the cells by centrifuging. The substrate solution at 0.125 M (1 ml.), adjusted to pH 7.8 by the addition of 0.1 or 1.0 M NaOH, was placed in a 2.5 ml. volumetric flask. The solutions were buffered by the addition of 0.75 or 1 ml. of diethyl barbiturate (veronal) buffer (0.1 M, pH 7.8). For glycyl-glycine, 0.25 ml. of 0.01 M $CoCl_2$ was added, and for L-leucinamide and glycyl-L-proline 0.25 ml. of 0.01 M $MnCl_2$; these metal ions are required for the manifestation of the full activity of these enzymes (19). The enzyme which splits the tripeptide does not require the addition of metal ions, and, in the absence of metal ion, the hydrolysis ceases after the complete splitting of one peptide bond. After incubating the mixture at 40° for 15 minutes, 0.5 ml. of serum was added to each flask to make a final volume of 2.5 ml. The final substrate concentration was 0.05 M in each instance. Samples (0.2 ml.) were withdrawn periodically from the incubating mixture and were titrated for the presence of free carboxyl groups by the alcohol method of Grassmann and Heyde (22), with standardized 0.01 M NaOH in 90 per cent ethanol. In general, samples were taken immediately after adding the serum, at 1 or 2 hour intervals during the first 8 hours, and subsequently at 24 and 48 hours.

The hydrolysis of all the substrates is reasonably described by the first order equation, $K = 1/t \log (100/100 - x)$, where x is the percentage of the substrate hydrolyzed in time t given as minutes, in decimal logarithms. Some of the detailed data are given in Table I.

For the dog experiments, control samples were taken 3 days before and immediately prior to injection of 1 gm. of phenylhydrazine hydrochloride intraperitoneally. Dog D weighed 12.5 kilos and Dog R 25.0 kilos. The volume of packed red cells and the percentage of reticulocytes were determined by the usual methods (23). The serum hemoglobin was determined by the method of Flink and Watson (24). The total serum bilirubin was estimated by the procedure of Malloy and Evelyn (25).

The few data shown in Table I indicate that the level of the enzymes

which hydrolyze diglycylglycine (GGG) and glycylglycine (GG) increases markedly following the injection of phenylhydrazine. It is also apparent that the several values of K determined for each sample are fairly well

TABLE I

Effect of Phenylhydrazine Anemia on Hydrolysis of Peptides by Dog Serum

Representative data for the hydrolysis of peptides of 0.2 ml. of serum per ml. of incubation mixture. K is the first order velocity constant in decimal logarithms. The complete data for the variation in K with time after injection of phenylhydrazine are shown in Table II.

Dog	Substrate	Time following injection	Time	Hydrolysis	$K \times 10^4$	Average $K \times 10^4$
		days	*hrs.*	*per cent*		
D	Diglycylglycine	−3	2	33	14	
			3	36	11	
			4	49	12	
			5	57	12	12
		1	0.5	14	22	
			1	27	22	
			1.5	43	27	
			2	48	23	
			2.5	66	31	25
		4	0.5	23	38	
			1	40	37	
			1.5	48	32	
			2	70	44	38
D	Glycylglycine	−3	22	25	0.9	
			46	43	0.9	0.9
		2	2	8	3.1	
			3	11	2.7	
			5	21	3.5	
			21	52	2.5	3.0
			46	102		
R	Glycyl-L-proline	−3	3	35	10	
			4	43	10	
			5	49	10	10
		4	2	33	15	
			4	50	12	
			5.5	58	11	12.3

reproducible. The changes produced by the anemia are certainly far above any individual variation encountered.

The detailed data given in Table II show that with both enzymes this increased level is maintained for some time but finally returns to normal at about the same time that the blood picture also becomes normal. While there is some day to day variation in the enzyme pattern, there can be

little doubt of the great increase in the serum levels of the tripeptide- and dipeptide-splitting enzymes, and that this occurs at about the same time as the high level of serum hemoglobin.

TABLE II

Effect of Phenylhydrazine Anemia on Serum Peptidases

After obtaining the samples on Day 0, each animal was given 1 gm. of phenyl-hydrazine hydrochloride intraperitoneally. The peptidase values are the first order velocity constants ($K \times 10^4$) for 0.2 ml. of serum per ml. of test solution.

Dog	Day	GGG	GG	LA	GP	Protein N	Volume of packed red cells	Reticulo-cytes	Serum hemo-globin	Serum bilirubin
						mg. per ml.	*ml. per 100 ml.*	*per cent*	*mg. per cent*	*mg. per cent*
D	−3	12	0.9	0.7	8		51			
	0	11	0.9	0.6	11	12.8	50	1.4	29	0
	1	25	2.3	2.4	12	10.8	24	2.0	495	
	2	19.5	2.8	2.1	16	6.0	24	9.0	600	0.31
	4	38	1.8	0.8	7	8.5	26	15.4	352	0.57
	5	31	4.2	2.1	11	9.5	21	19.0	210	0.80
	6	26.5	1.4	1.4	10	10.0	20	30.4	11	0.38
	7	24	2.3	1.6	10	10.7	24	43.0	26	
	8	27	0.9	1.1	6	8.5	25	33.8	8	0
	9	31		1.8	8	9.6	28	39.8	20	0.25
	11	29	0.6	0.8	7	8.2	29	18.3	46	0.09
	13	26	1.2	1.7	8	10.4	32	16.0	29	0.13
	19	8	0.5	0.8	9	9.3	42	3.0	8	0.16
	27	9	0.7	0.7	11		46	1.4	17	0.13
R	−3	14	1.0	0.8	10		46		83	0.09
	0	12	0.7	0.4	14	12.8	47	1.0		
	1	51	1.4	0.6	14	9.5	31	1.2	165	0.25
	2	43	1.6	0.9	12	10.4	29	1.6	197	0.25
	4	19	2.2	0.7	12	11.2	32	2.8	14	0.31
	5	20	2.0	1.4	11	10.3	33	6.0	24	0.31
	6	18		1.0	10	10.2	33	13.4	38	0.16
	7	24.5	1.8	1.2	11	9.6	38	17.2	42	0.19
	8	22	1.6	0.5		8.9	32	14.8	20	0.13
	9	21	0.8	1.1	9	10.5	36	5.8	66	0.22
	11	15	0.5	0.9	10	8.5	36	2.8	18	0.25
	13	10	0.8	0.4	9	10.5	37	3.0	19	0
	19	12.5	0.8	0.6	10	9.1	44	0.6	24	0.06
	27	11	0.8	0.7	10		46	0.2	14	0.28

The low level of leucine aminopeptidase (LA) of the normal dog was significantly increased in the anemic dogs, but considerable variation is encountered. On the other hand, prolidase (GP) is present in the serum of normal dogs at a reasonably high level, but no significant changes were found following the injection of phenylhydrazine.

Alterations in the volume of red cells and the reticulocyte response were those usually encountered in the production of an anemia of this type.   The increased bilirubin and the presence of high levels of serum hemoglobin show that extensive destruction of erythrocytes had occurred.

As already mentioned in our introductory remarks, it is quite clear from the evidence given here and that presented by others that certain serum peptidases must originate in large part from the breakdown of erythrocytes.   The extreme variations in tripeptidase, glycylglycine dipeptidase, and leucine aminopeptidase in serum are in contrast to the marked constancy of prolidase which is not significantly altered by conditions which affect other peptidases.   These findings suggest that prolidase may originate from cells other than erythrocytes, and that erythrocytes probably

TABLE III

Effect of Injection of Lipoadrenal Cortex on Serum Peptidases

These tests were performed in the manner already described.   The results are expressed as $K \times 10^4$, the first order velocity constant for 0.2 ml. of serum per ml. of reaction mixture.   The sample marked as zero time was taken immediately before the infusion of the steroids.

Time after injection of steroids	GG		GGG		LA		GP		Lymphocytes per c.mm.	
	Dog D	Dog R	Dog D	Dog R	Dog D	Dog R	Dog D	Dog R	Dog D	Dog R
hrs.										
0	0.8	0.7	14	14	0.7	0.7	8.5	9.3	3400	3200
6	0.9	1.0	16	16	0.9	0.9	8.2	8.4	1950	2550
9	0.7	0.7	12	14	0.7	0.5	8.4	6.9	2800	2000
24	0.6	0.6	16	14	0.9	0.4	8.3	8.1	1350	2600
96									4600	5900

contain little or no prolidase.   This possibility will be investigated further.

Effect of Injection of Adrenal Extracts—In order to determine whether the peptidases might not have been released from erythrocytes or from the simultaneous breakdown of lymphocytes, dogs were treated with adrenal steroids.   The same animals (Dogs D and R) in which anemia had been produced by phenylhydrazine were used following their complete recovery.   They were injected with 0.5 ml. of lipoadrenal cortex (Upjohn)[1] per kilo of body weight.   Dog R received 12.5 ml. subcutaneously, and Dog D 7.0 ml.   The data in Table III show that a significant decrease in circulating lymphocytes was produced.   However, no alteration occurred

[1] We are indebted to Dr. John F. Norton of The Upjohn Company, Detroit, Michigan, for the supply of cortical extracts.

in the peptidase levels. An additional test was made on a third animal (Dog C) which was given 60 ml. of aqueous cortical extract[1] intraperitoneally. Again the lymphocyte level was greatly reduced but no changes occurred in the activity of the peptidases (Table IV).

It is extremely important to note how constant the serum peptidase levels are in normal animals, as demonstrated by the data in Tables III and IV. These experiments serve as additional controls and give further emphasis to the significance of the peptidase alterations encountered in the animals with experimentally produced hemolytic anemia.

Samples of serum taken from Dog C prior to the administration of the aqueous cortical extract and samples obtained 6 and 24 hours later were examined electrophoretically. The runs were performed in 0.1 M veronal buffer at pH 8.4 and at a protein concentration of 1.5 per cent. No sig-

TABLE IV

Effect of Aqueous Cortical Extract on Serum Peptidases

The data are given as $K \times 10^4$, the first order velocity constant for 0.2 ml. of serum per ml. of reaction mixture.

Time after injection of steroids	GG	GGG	LA	GP	Lymphocytes per c.mm.
hrs.					
−24					4300
0	1.0	12	1.2	11	3700
6	0.6	11	0.8	10	1700
9					1000
12	0.7	12	0.7	11	3000
24	0.5	12	0.7	10	2000

nificant variation of the relative distribution of the serum proteins was found. Although there have been conflicting claims regarding serum protein alterations produced by injection of cortical extracts in some species (26), these changes have not been observed in man (12, 26). Our observations on a single animal suggest that the dog resembles the human in this respect.

Human Serum Peptidases—Prior to our realization that the serum peptidases might originate from the breakdown of erythrocytes, we had proceeded to amass considerable data on the peptidases of human sera from normal individuals and from patients with a variety of metabolic abnormalities. In fact, it was the observations on samples of sera from a patient with hemolytic anemia which led to the dog experiments.

Some representative data are given in Table V. The studies made on patients with hemolytic anemia are still not clear. We have now studied three acute cases; in two of these the serum peptidase levels were within

the rather wide normal range, but one (Individual 6) showed remarkably high levels of activity towards GGG and LA. The values obtained on samples of serum taken 2 days apart do not differ significantly. In one of the two cases of congenital hemolytic jaundice (Individual 9) and the single case of pernicious anemia which were examined, the peptidase values as measured by GGG and LA seemed to be slightly elevated. A number of sera from individuals with progressive muscular dystrophy (both childhood and facioscapulohumeral types (27)) also showed quite normal values. Our experience thus far does not point to any simple correlation

TABLE V

Level of Human Serum Peptidases

The data for the peptidase activities are presented as $K \times 10^4$ for 0.2 ml. of serum per ml. of reaction mixture.

Individual	GG	GGG	LA	GP
1. Normal	2.2	3.1	0.3	4.6
2. "	4.5	3.0	0.5	2.6
3. "	2.0	6.2	0.7	3.0
4. "	2.3	4.2	0.3	2.0
5. "	1.3	5.3		6.7
6. Acute hemolytic anemia (acquired)	5.0	34	19	6.1
	3.6	31	13	6.5
7. " " " "	1.9	2.4	0.5	1.8
	2.0	2.6	0.5	1.6
8. " " " "	4.5	2.0	0.4	2.2
9. Congenital hemolytic jaundice	2.6	8.8	1.0	2.0
10. " " "	0.5	2.3	0.2	3.0
11. Pernicious anemia	1.7	8.1	1.2	5.0
12. Muscular dystrophy (childhood)	2.0	2.1	0.5	2.5
13. " " "	1.7	4.2		2.9
14. " " "	3.7	3.2		3.9
15. " " (facioscapulo-humeral)	3.8	3.4	0.5	1.0

of serum peptidase level and metabolic state. Consequently we are refraining from presenting further data at this time. In view of the recent interest in these serum enzymes and the announced intention of pursuing such studies (7, 15, 18), the present information is given mainly to call attention to the one instance where strikingly high peptidase values were found.

SUMMARY

1. It has been found that anemia in dogs produced by the administration of phenylhydrazine was associated with a marked increase in serum

peptidases concurrent with the breakdown of erythrocytes. This suggests that, in the dog, serum peptidases probably originate in large part from the dissolution of erythrocytes.

2. Injection of cortical extracts in dogs produced a marked lymphopenia but had no detectable effect on the serum peptidase levels.

3. Some data are presented concerning the activity of peptidases in human sera. One patient with acute hemolytic anemia showed a very high serum peptidase level. However, in other cases there was little or no change from the values found in normal sera.

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THE CONVERSION OF N¹⁵-CONTAINING INDOLE TO NIACIN BY NIACIN-REQUIRING STRAIN 39401 OF NEUROSPORA*

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Since the observation by Krehl *et al.* (1) that tryptophan can replace niacin in growth experiments with rats, a great deal of work has been carried out in an attempt to elucidate more fully the nature of this relationship. Much of our knowledge of the possible mechanism involved in this conversion stems from investigations with mutant strains of *Neurospora*. Several distinct genetic strains of *Neurospora* which require niacin for growth are known. The biochemical differences found between these strains indicate that tryptophan may serve as a niacin precursor, and suggest a possible mechanism for this conversion (2–6). That tryptophan is involved in niacin synthesis is suggested primarily by strains of one genetic type, representative of which is strain 39401. This strain requires niacin, yet will grow when given hydroxyanthranilic acid, kynurenine, tryptophan, or indole (2–5). Strains are also known which require niacin or hydroxyanthranilic acid for growth but cannot utilize kynurenine, tryptophan, or indole (5). Still other strains can grow only on niacin, yet accumulate hydroxyanthranilic acid (strain 4540) (7) or quinolinic acid (strain 3416) (6). These various biochemical differences between the niacin-requiring mutant strains suggest that tryptophan serves as a precursor of hydroxyanthranilic acid, which in turn is converted to niacin, as summarized in Fig. 1.

The scheme given in Fig. 1, however, does not satisfactorily account for strain 39401, since no provision is made for the tryptophan synthesis that occurs when this strain is grown on niacin. Since strain 39401 differs from the parental strain by alteration of a single gene (2), an orthodox interpretation is then that this strain is blocked in a single biochemical reaction. Inasmuch as strain 39401 can grow on niacin, hydroxyanthranilic acid, kynurenine, tryptophan, or indole, but not on anthranilic acid, a presumed precursor of indole in *Neurospora* (8, 9), one would conclude that the defective biochemical step characteristic of this strain is concerned with the synthesis of indole. Such a conclusion, however, is at variance with the observation that when this strain is grown on niacin the formed

mycelium contains tryptophan. Tryptophan synthesis obviously occurs, and the quantity of niacin required to produce a given amount of mycelium is insufficient to account for the amount of tryptophan in the mycelium if niacin served as a precursor. A second difficulty encountered in the foregoing interpretation is the fact that there are mutant strains which are blocked in the synthesis of indole but cannot utilize anthranilic acid, and yet cannot utilize either kynurenine or niacin (10, 5). Still further observations have suggested that tryptophan may not necessarily serve as a precursor of hydroxyanthranilic acid, since double mutant strains combining strain 39401 and a strain which accumulates hydroxyanthranilic

Fig. 1. Proposed scheme for the conversion of indole to niacin in *Neurospora*, based on work of Beadle *et al.* (3), Bonner *et al.* (2, 5, 6), and Mitchell and Nyc (4). Genetic blocks indicated by strain number.

acid do not accumulate this substance (5). A similar situation occurs with regard to quinolinic acid accumulation in double mutant stocks formed between strain 39401 and a quinolinic acid-accumulating strain.

It is known that gene mutations in *Neurospora* may lead to loss of biochemical function in an indirect manner (11, 12), since it is possible that intermediates or their metabolites, accumulated as a result of genic alteration, may in turn prove antagonistic to other cellular reactions. The observations regarding strain 39401 outlined above suggest the possibility that the relationship between niacin and tryptophan in strain 39401 may be indirect and that tryptophan may in an indirect manner circumvent the genetic block characteristic of this particular mutant. Since the scheme given in Fig. 1 is predicated on the ability of strain 39401 to grow on added tryptophan, it seemed desirable to check this conversion with a

marked atom in indole that might reasonably be expected to be found in niacin. The nitrogen atom of the indole ring would fulfil this requirement, since it should appear as the nitrogen of the pyridine ring of niacin. The conversion of indole containing an excess of N^{15} has therefore been investigated.

<div align="center">EXPERIMENTAL</div>

Mutant strain 39401 was used for this experiment. Indole having 33.1 atom per cent excess N^{15} was used (13). 19 liters of minimal medium (14) contained in a 5 gallon Pyrex carboy were prepared and sterilized; 15.9 mg. of N^{14}-indole and 20.3 mg. of N^{15}-indole having 33.1 atom per cent excess N^{15} were dissolved in 10 cc. of 50 per cent ethanol and added to the hot medium. After cooling, the medium was inoculated with strain 39401 and maintained at 25° with forced aeration for 7 days. The mycelium was then removed by filtration through cheese-cloth, squeezed dry, and lyophilized. 19.5 gm. of dry mycelium were so obtained.

The dry mycelium, thoroughly ground, was shaken with 200 cc. of 1 N H_2SO_4 for 1 hour at 25°. It was then pressed dry in a Büchner funnel lined with cheese-cloth. The extraction was repeated by shaking the mycelium with 100 cc. of 1 N H_2SO_4 for 1 hour and filtering as before with a final wash with a 50 cc. portion of 1 N H_2SO_4. The combined filtrates were then autoclaved at 15 pounds for 40 minutes. 1200 γ of niacin were found in this extract with the standard *Lactobacillus arabinosus* assay (15).

This extract was next treated with Lloyd's reagent. 30 gm. of Lloyd's reagent were added in two portions, with filtration between additions. The filtrate was found to contain little niacin (60 γ) and was discarded. After washing with water, the combined Lloyd's reagent was treated with ammonium hydroxide to elute the niacin. This treatment was carried out by boiling for 5 minutes with each of several successive portions of 5 N NH_4OH. 1.5 cc. of 4 N NaOH were added to the combined filtrates and washings, and the resulting solution was boiled gently until the vapors were no longer alkaline to litmus. Assay of this eluate indicated the existence of 880 γ of niacin.

Since the eluate was highly colored, it was passed through a 15 cm. column of Amberlite IRC-50 resin (1 cm. in diameter) which had been put in the acid cycle by washing with 1 N HCl and then with water until the eluate was free from chloride ion. The niacin extract was allowed to flow through the column, which was then washed with 65 cc. of distilled water at the same rate (1.5 cc. per minute). No loss of activity was encountered in this step. The effluent was lyophilized. The dry residue was shaken for 10 minutes with 4 cc. of water, filtered, relyophilized, and the dry residue extracted with a total of 15 cc. of 95 per cent ethanol. The resulting lightly colored extract was found to contain 420 γ of niacin.

To free this small amount of niacin from the remaining impurities the entire alcohol-soluble fraction was applied to a single sheet of Whatman No. 1 filter paper by the method of Yanofsky *et al.* (7) and developed as an ascending chromatogram, with 85 per cent butanol, 4 per cent formic acid, and 11 per cent water as the developing solvent. The position of the nicotinic acid was determined by applying a narrow lengthwise section of the paper chromatogram, sterilized by ultraviolet light, to a large poured plate (16) of agar medium containing no niacin, inoculated with *L. arabinosus*. The plate was kept at 37° for 12 hours, at which time a zone of turbidity was readily visible. The cross section of the chromatogram, corresponding to the zone of turbidity found on the plate, was cut out and eluted by shaking twice with 25 cc. of 95 per cent ethanol. Assay of this eluate gave a total of 158 γ of niacin.

Since at this point the niacin was virtually pure, 8.75 mg. of N^{14}-niacin were added to permit isolation. The resulting solution was evaporated to dryness and the residue sublimed twice. The melting point of the nicotinic acid so obtained was 225° uncorrected. 3.6 mg. of nicotinic acid were recovered, and were diluted 4-fold with N^{14}-nicotinic acid for isotope determination. The actual found value on analysis of this material was 0.021 atom per cent excess N^{15}. Since a 226-fold dilution was made in the course of this procedure, 56.5-fold after chromatographic separation of the niacin and 4.0-fold for analysis, the calculated excess N^{15} per cent in the niacin isolated from the formed mycelium is 4.7 atom per cent excess.

DISCUSSION

From the experiment just described it is apparent that the nitrogen of the indole used for growth of strain 39401 is found in the pyridine ring of the formed niacin. Thus indole serves as a precursor of niacin in this strain. Since indole gives rise to tryptophan in *Neurospora* (9), it may also be concluded that tryptophan is probably likewise serving as a niacin precursor. These results confirm in *Neurospora* the work carried out on rats by Heidelberger *et al.* (17, 18) with C^{14}-marked tryptophan, and by Schayer (13) with N^{15}-marked tryptophan.

The quantitative aspects of this experiment, however, are perhaps of greater interest. If one assumes that this mutant is blocked in the synthesis of indole, the formed niacin should reflect nearly the same excess N^{15} as was present in the indole used to grow the culture. The fact that only 4.7 atom per cent excess N^{15} is found in the isolated niacin, whereas 18.6 atom per cent excess N^{15} was present in the indole used to grow the strain, suggests that only about one-fourth of the isolated niacin was directly formed from the added indole. The figure of 4.7 atom per cent excess is of course subject to error. At only two points in this procedure,

however, might serious errors be expected, *i.e.* in the niacin determination after chromatographic separation and in the N^{15} determination. The expected errors in these determinations do not account for the discrepancy from the predicted value. The conclusion must then follow that the niacin formed from the added indole is diluted with niacin formed from some other source.

Various interpretations regarding the source of this extra niacin can be made. As discussed in the introductory section of this paper, strain 39401 is known to synthesize tryptophan when grown on niacin. A reasonable explanation of the extra niacin might then be that the niacin formed from the added indole in turn gives rise to tryptophan, which in turn gives rise to further niacin. A second interpretation might be that niacin synthesis from a non-indole source can occur in the presence of indole or a metabolite of indole. A third possibility is that the nitrogen is exchanged with other cellular constituents at some point in the formation of niacin. The validity of any one of these interpretations can be decided only by similar experiments with other mutant strains, which work is being carried out. Thus, while one may conclude that indole serves as a niacin precursor in strain 39401, the identification of the source of the extra niacin synthesis as well as the characterization of the genetically blocked reaction characteristic of this strain must await further work.

SUMMARY

A mutant strain of *Neurospora* 39401 is known from the work of several investigators to require niacin for growth and to be able to utilize 3-hydroxyanthranilic acid, kynurenine, tryptophan, or indole in place of niacin. As a direct check of the conversion of indole to niacin by this strain, it was grown on indole containing 18.6 atom per cent excess N^{15}. The niacin present in the formed mycelium was isolated and analyzed for its N^{15} content, which was found to be 4.7 atom per cent excess. The implications of the discrepancy between the observed value of excess N^{15} present in the isolated niacin and the predicted value of 18.6 atom per cent excess are discussed.

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PREPARATION AND CHARACTERIZATION OF THE ENZYME WHICH CONVERTS TESTOSTERONE TO ANDROSTENEDIONE*†

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Since the destruction of testosterone by liver mince (1) is accelerated by the addition of either diphosphopyridine nucleotide (DPN) or citrate (2), an attempt has been made to obtain active cell-free extracts from liver tissue and to isolate the different enzyme systems involved. In this paper the separation of the enzyme activated by DPN which converts testosterone to androstenedione is described and its physicochemical characteristics determined.

EXPERIMENTAL

The analytical and incubation procedures used were those developed by Samuels (3) and Samuels, McCaulay, and Sellers (1). The α,β-unsaturated ketone structure of testosterone was measured spectrophotometrically at 240 mμ and the 17-ketosteroid group was determined by the Callow, Callow, and Emmens (4) modification of the Zimmermann reaction (5). The DPN used was obtained from the Schwarz Laboratories. Infra-red spectra were determined on the Beckman IR2 spectrophotometer.

Preparation of Extracts—Steer liver tissue was cut into 1 cm. slices and packed in chipped ice immediately after being removed from the animal. The tissue was then ground in a cold meat grinder and 1 kilo was placed in a flask containing 1 liter of water at 2–4°. After standing for 24 hours with occasional stirring, the material was filtered through several layers of cheese-cloth and centrifuged in a refrigerated centrifuge for half an hour at 800 $\times$ g. The microscopic cell fragments were then removed from the supernatant by centrifuging 1 hour at 20,000 $\times$ g.

The resulting preparation consisted of a slightly opalescent red supernatant fluid and a gray residue. To test for enzyme activity, aliquots of the extract and precipitate equivalent to 2 gm. of fresh liver were incubated in buffer solution at pH 7.4 with 7 mg. of diphosphopyridine nucleotide and 200 γ of testosterone as previously described (1). All significant activity was in the supernatant fluid. This preparation contained 72 mg. of solids per ml.

To investigate the possibility of other diffusible cofactors, this supernatant solution was subjected to dialysis against cold, constantly chang-

TABLE I

Enzymic Activity of Preparations from Steer Liver on Rings A and D of Testosterone

Preparation	Conjugation in ring A destroyed (testosterone per flask)	17-Ketosteroids formed (androstenedione per flask)	Enzyme solids per flask
	γ	γ	*mg.*
Centrifuged extract, 20,000 $\times$ g for 1 hr.			
Before dialysis	82	79	360
Dialyzed extract	27	73	
0.5 saturation with $(NH_4)_2SO_4$			
Supernatant	(11)	75	24.5
Ppt.	(6)	(6)	
Complete saturation with $(NH_4)_2SO_4$			
Supernatant	(11)	0	
Ppt.	(6)	56	

All flasks contained 20 ml. of a buffer solution, pH 7.4, containing KCl 0.0056 M, $MgCl_2$ 0.0021 M, NaCl 0.08 M, Na_2HPO_4-NaH_2PO_4 0.04 M, and extract equivalent to 2 gm. of fresh liver tissue. The flasks were incubated at 38° for 3 hours after being filled with oxygen.

The figures in parentheses are within the limits of experimental error and are therefore insignificant.

ing, distilled water for 72 hours. After this time a flocculent precipitate formed which was easily separated by low speed centrifugation. The resulting supernatant was bright red and sparkling clear. As with the crude extracts, all significant activity remained in solution. A comparison of the testosterone-destroying activity of this preparation with those of various others can be seen in Table I. It will be noted that the activity of the 17-ketosteroid-forming system per unit weight of solids increased upon purification, while the ability of the preparations to destroy the α,β-unsaturated ketone group tended to disappear.

To effect further purification of the 17-ketosteroid-forming enzyme, two portions of this clear extract were treated with ammonium sulfate

to give half and fully saturated solutions respectively. These preparations were then centrifuged and dialyzed for 72 hours or until tests for the sulfate ion were negative. As will be seen in Table I, the 17-ketosteroid-forming enzyme remained dissolved in the 0.5 saturated ammonium sulfate solution without loss of activity, but was precipitated from the saturated solution with approximately one-third loss in activity. Since the 17-ketosteroid enzyme was still in solution after dialysis and was not precipitated by half saturation with ammonium sulfate, it would appear that the enzyme molecule is a relatively simple protein similar to the albumins.

The findings of Hogeboom *et al.* (6) and Claude (7) raised the question of whether the 17-ketosteroid-forming enzyme in the distilled water extract had been released from some particulate fraction of the cell during the process of extraction. To examine this possibility an 0.88 M sucrose extract was prepared. After grinding, 1 kilo of liver was added to 1 liter of 1.5 M sucrose solution. On the basis of 70 per cent water in the liver tissue this gave a final concentration of 0.88 M sucrose. The extract was then prepared by high speed centrifugation, dialysis, and fractionation with 0.5 saturated ammonium sulfate in the same way as the aqueous extract. This solution had only a faint pink color in contrast with the deep red color of the water extract.

When 5 ml. each of the sucrose and distilled water extracts were tested for enzymatic activity, 50.8 and 51.8 γ of 17-ketosteroids respectively were formed in 1 hour. The solid matter was 5.5 mg. per ml. in the purified sucrose extract and 4.9 mg. per ml. in the final aqueous preparation. This evidence, together with the observation that there was no significant activity in the granules sedimented at high speed centrifugation, is a strong indication that the enzyme is from the cytoplasmic solution in the cell. As there was less cytochrome *c* in the sucrose extracts, it would appear that cytochrome *c* is not a limiting factor in the reaction.

Isolation of Product—To identify the products formed in the DPN-activated reaction, 2 mg. of testosterone were incubated with 100 ml. of ammonium sulfate-fractionated extract in 200 ml. of phosphate buffer at pH 7.8 and 33° for 3 hours. The mixture was boiled and extracted with ether, and was subsequently evaporated to dryness.

The residue from the ether extraction was dissolved in hexane and adsorbed on a column of ignited aluminum oxide, from which it was eluted with 10 per cent chloroform in hexane in small fractions by use of the Technicon automatic fractionating apparatus. 40 drops of the solvent were collected in each tube.

All the steroid hormones detectable with the ultraviolet spectropho-

tometer or the Zimmermann reaction were eluted in the first fifty tubes. Tubes 18 to 24 and 47 to 49 showed the absorption at 240 mμ characteristic of α,β-unsaturated ketones. The Zimmermann reaction showed that the 17-ketosteroid was eluted in the first series of tubes showing α,β-unsaturation. All others, including Tubes 47 to 49, gave negative reactions.

The α,β-unsaturated 17-ketosteroid had a melting point of 171.5–174.5° and a mixed melting point with a known sample of Δ^4-androstene-3,17-dione (m.p. 172–174°) of 170–174°. An infra-red spectrum was
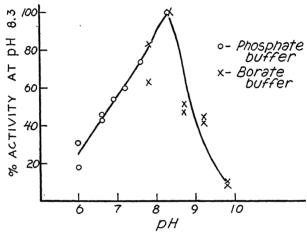
FIG. 1. Effect of pH on the formation of 17-ketosteroids from testosterone by liver extract. Each flask contained 10 ml. of dialyzed supernatant after fractionation with 0.5 saturated $(NH_4)_2SO_4$, 10 ml. of 0.08 M buffer, 7 mg. of DPN, and 0.7 μM of testosterone. Incubation was at 37° for 3 hours in O_2. Since the activity of the preparation used with borate buffer differed somewhat from that used in the experiment with phosphate buffer, the activity at pH 8.3 has been taken as 100 per cent.

identical with that of a known sample of Δ^4-androstene-3,17-dione. The unsaturated, non-17-ketosteroid was shown to be unchanged testosterone. It was estimated that over 1 mg. of androstenedione was formed and somewhat less than 1 mg. of testosterone remained unchanged.

The relatively good recovery of Δ^4-androstene-3,17-dione in comparison to the amount calculated to be formed, together with the comparable recovery of testosterone, showed Δ^4-androstene-3,17-dione to be the major product of the reaction.

Properties of Enzyme Oxidizing Carbon 17—The effect of pH on the enzyme forming 17-ketosteroids was investigated with sodium phosphate buffers over the range of pH 6 to 7.8 and borate buffers over the range
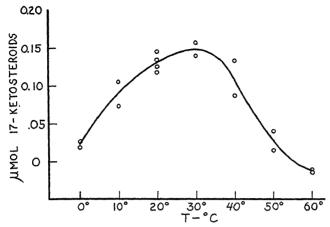
FIG. 2. Effect of temperature on formation of 17-ketosteroids from testosterone by liver extract. Each flask contained 10 ml. of dialyzed supernatant after fractionation with 0.5 saturated $(NH_4)_2SO_4$, 10 ml. of 0.08 M phosphate buffer (pH 7.4), 7 mg. of DPN, and 0.7 μM of testosterone. Incubation was at 37° for 3 hours in O_2.
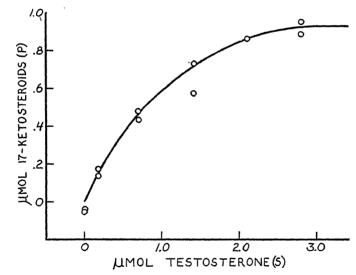
FIG. 3. Effect of concentration of testosterone on formation of 17-ketosteroids by liver extract. Each flask contained 10 ml. of dialyzed supernatant after fractionation with 0.5 saturated $(NH_4)_2SO_4$, 10 ml. of 0.08 M phosphate buffer (pH 7.8), and 7 mg. of DPN. Incubation was at 33° for 3 hours in O_2. S = substrate; P = product of reaction.

of pH 7.8 to 10. Fig. 1 shows the pH optimum of the enzyme to be in the region of pH 8.3.

The enzyme was incubated at temperatures of 0°, 10°, 20°, 30°, 40°, 50°, and 60° for 3 hours in the presence of DPN. As seen in Fig. 2, the optimum temperature for the enzyme is between 30–40°. The lower
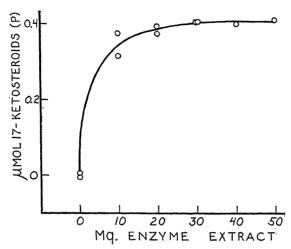
FIG. 4. Effect of enzyme concentration on formation of 17-ketosteroids from testosterone by liver extract. Each flask contained a total volume of 20 ml. containing 0.04 M phosphate buffer (pH 7.8), 7 mg. of DPN, and 0.7 μM of testosterone. Incubation was at 33° for 3 hours in O_2. The extract used was the dialyzed supernatant after fractionation with 0.5 saturated $(NH_4)_2SO_4$ and contained 5 mg. of solids per ml. P = product of reaction.

TABLE II

Effect of Various Buffers on 17-Ketosteroid-Forming Enzyme

Incubation medium	pH	17-Ketosteroids formed per flask
		γ
Physiological buffer solution*...........	7.8	117
0.04 M sodium borate buffer............	7.8	120
0.04 " " phosphate buffer........	7.8	128

The flasks all contained a final volume of 20 ml. and were incubated at 33° for 3 hours. Each flask contained 5 ml. of the ammonium sulfate-purified extract and 200 γ of testosterone and was filled with oxygen.

* The physiological buffer solution contained KCl 0.0056 M, MgCl$_2$ 0.0021 M, NaCl 0.08 M, Na$_2$HPO$_4$-NaH$_2$PO$_4$ 0.04 M.

value at 40° would indicate that the optimum temperature is nearer 30°. There is definite activity at 0°, but none at 60°.

Fig. 3 shows that, the enzyme concentration being constant, more 17-ketosteroids are formed as the concentration of the substrate rises.

The leveling of the curve is probably due to the saturation of the enzyme.

In the experiment illustrated in Fig. 4, the effect of enzyme concentration on the rate of 17-ketosteroid formation was investigated. Apparently the amounts of enzyme used in studies on pH and temperature were not limiting factors. Concentrations of standard extracts of 30, 40, and 50 mg. (dry weight basis) per flask, which represented 6, 8, and 10 ml. of extract respectively, were all on the plateau of the curve. Concentrations of 10 and 20 mg. appear to be on the ascending arm.

Table II shows that the 17-ketosteroid enzyme system does not seem to require any particular ion or metal added to the incubation medium for its activity. This does not, however, exclude the possible presence of a bound, non-diffusible, metal complex in the enzyme preparation itself. The fact that incubations with the Krebs-Ringer's solution and simple borate and phosphate buffers gave comparable activities excludes the necessity of the ions in amounts ordinarily used in physiological salt solutions.

DISCUSSION

The action of DPN depended upon the state of purity of the preparation. DPN in minces (2) and crude extracts increased the destruction of the α,β-unsaturated ketone groups and enhanced the formation of 17-ketosteroid groups, while in the more purified extracts only the group on carbon atom 17 was affected. A possible explanation of this observation could be that an enzyme acting on the conjugated system in ring A works more effectively on a compound having a ketone group on carbon atom 17, such as androstenedione, than on a 17-hydroxy compound. Thus, when DPN forms a 17-ketosteroid, it may be producing a compound more efficiently metabolized by the second mechanism.

It is probable, therefore, that testosterone may be primarily attacked at either ring A or D, depending upon the enzymes and cofactors available; nevertheless, as DPN increases both the breakdown of the α,β-conjugated ketone system and the formation of 17-ketosteroids, it would appear that the favored route is through Δ^4-androstene-3,17-dione formed by the DPN-activated enzyme.

Kochakian and coworkers (8, 9) first isolated Δ^4-androstene-3,17-dione from incubation of testosterone with liver slices. They also introduced evidence of the reversibility of this system. Their yields of the diketone were relatively small, however, probably because the system was neither reinforced with DPN nor protected from the action of nucleotidases.

Several workers have suggested that this compound holds a key position in the series of reactions between testosterone and androsterone. Of the three most likely pathways of intermediary metabolism proposed by

Koch (10) our studies with DPN would lend the greatest support to his third scheme which suggests Δ^4-androstene-3,17-dione as the first product formed from testosterone. His other two schemes propose androstane-17β-ol-3-one and Δ^5-androstene-3,17-diol as the first products of metabolism of testosterone and are, therefore, not consistent with our findings in the DPN-activated system.

Since the 17-ketosteroid enzyme was still in solution after dialysis and was not precipitated by half saturation with ammonium sulfate, it would appear that the enzyme molecule is similar to the albumins.

Certain useful conclusions can be drawn from the incomplete kinetic data presently available. Under conditions involving excess cofactor a modified Michaelis-Menten reaction scheme (11) is probably applicable.

$$EC + S \xrightleftharpoons[k_2]{k_1} ECS \xrightarrow{k_3} P + EC$$

C = oxidized DPN; E = enzyme; S = testosterone; P = androstenedione. For present purposes an integrated form of the familiar rate-velocity expression,

$$v = \frac{k_3 e [S]}{[S] + \dfrac{k_2 + k_3}{k_1}}$$

is required.

$$\frac{([S]_1 - [S]_2)}{(t_2 - t_1)} = k_3 e + \frac{K_m \ln\left(\dfrac{[S]_2}{[S]_1}\right)}{(t_2 - t_1)}$$

where v = velocity, t = time, $[S]$ = concentration of testosterone, and $K_m = (k_2 + k_3)/k_1$. The data plotted according to this equation are given in Fig. 5. They yield the values $k_3 e = 5.7 \times 10^{-9}$ mole per second-liter; $K_m = 3.3 \times 10^{-5}$ mole per liter. The data have been fitted by eye, and, since the number of determinations is small, the above parameters can only be considered approximate.

In Fig. 4 are plotted as ordinate the amounts of product formed in 3 hours against the concentrations of enzyme for an initial substrate concentration of 0.7 μM and as before an excess concentration of DPN. A K_m value of 3.3×10^{-5} mole per liter indicates that $k_1 = 3.0 \times 10^4$ $(k_2 + k_3)$; so that under the condition $[S] < e$, the substrate is very rapidly converted to ECS, which decomposes slowly with rate constant k_3 to products. The hyperbolic shape of Fig. 4 is therefore the result of the process whereby the concentration of substrate becomes rate-limiting.

At the point at which the reaction rate becomes independent of enzyme concentration the concentration of enzyme in the enzyme digest is approximately equal to $[S]/n$, where n is the number of active sites on the enzyme molecule. Determined in this way, the concentration of the enzyme is $(2.3 \times 10^{-8})/n$ mole per mg. of preparation, which yields a maximum molecular weight of 43,000, if one assumes that there is but one active center on the enzyme molecule. From the best value of k_3e calculated from the data of Fig. 5 and the total enzyme concentration calculated above for $n = 1$, $k_3 = 1.0 \times 10^{-4}$ sec.$^{-1}$. This may be compared
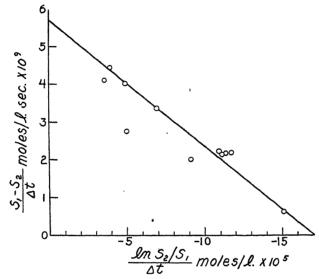
Fig. 5. Plot of Equation 2 from data from variation of substrate concentration (Fig. 3).

with the value calculated directly from Fig. 4, $k_3 = 0.7 \times 10^{-4}$ sec.$^{-1}$. Since the data of Fig. 4 were obtained at pH 7.4 and those of Fig. 5 at pH 7.8, which is closer to the optimum pH for enzymic activity, the agreement between two values of k_3 is more than satisfactory and may be interpreted as strong evidence in favor of the proposed mechanism.

SUMMARY

An enzyme oxidizing testosterone to Δ^4-androstene-3,17-dione has been prepared from steer liver. It appears to be in solution in the cytoplasm. Diphosphopyridine nucleotide is necessary as a hydrogen acceptor, but no metal has been found necessary for activation. Marked reduction in the cytochrome concentration of the extract did not affect its activity.

The enzyme is soluble in distilled water and half saturated ammonium sulfate. It is precipitated by full saturation. The optimum pH is 8.3 and the optimum temperature is 30–33°.

Analysis of the kinetic data indicates that the enzymic reaction in the presence of excess cofactor has a "Michaelis constant," K_m of 3.3×10^{-5} mole per liter. From this value and assuming only one center of enzymic activity per protein molecule, the molecular weight of the enzyme would be not more than 43,000. On this basis the specific reaction velocity constant, k_3, for the reaction forming final products is approximately 1×10^{-4} sec.$^{-1}$.

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ASSAY OF INSULIN IN VITRO BY FIBRIL ELONGATION AND PRECIPITATION

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When heated, a solution of insulin in acid (pH about 2.0) will form a gel, a precipitate or coagulum, or a mixture of the two. The precipitate is composed of flocs of spherites (1) whose subunits are radially oriented fibrils. Suspensions of dispersed fibrils (2) may also be prepared. In these, electron micrographs show that the fibrils have diameters of about 150 A and lengths of 15,000 to 40,000 A.

When one introduces fibrils formed at higher temperatures (80–100°) and cooled into solutions of native insulin at temperatures and pH values well within the stability range, the seeded fibrils will elongate at the lower temperature and quantitatively remove the native insulin from solution, thus transforming it into fibrous insulin (3). Elongation of insulin fibrils in the presence of native insulin has been found to take place in relatively impure solutions containing small amounts of insulin and in solutions which have been made acid by mineral acids, organic acids (for example, concentrations of acetic acid up to 50 per cent have been used), or mixtures. A quantitative recovery of insulin is often achieved, indicating that the elongation reaction may afford the basis for an assay of insulin *in vitro*. This paper describes a method *in vitro*, based on fibril elongation and precipitation.

Methods

Biological Assay—The sloped screen-mouse convulsion method devised by Thompson (4) was used with two 400 mouse assays. The results were calculated in terms of U. S. P. reference insulin run simultaneously with the samples. Experimental errors were determined by statistical analysis (5). The weighted mean values were obtained by weighting individual assays by the reciprocal of variance method.

Crude insulin solutions (still concentrates) were prepared for bioassay by adding 50 ml. of the sample to a mixture of 400 ml. of absolute alcohol and 400 ml. of commercial ethyl ether, and then stored at 5° overnight.

The precipitate was dissolved in 100 ml. of hydrochloric acid at pH 3.0. Appropriate dilutions of this solution were made for bioassay purposes.

Preparation of Initial Gel—A good quality crystalline pork insulin is dissolved in 0.05 N hydrochloric acid in 2 per cent concentration. 2 ml. aliquots of this solution are pipetted into annealed Pyrex vials which are then sealed. The tubes are immersed in boiling water for a period of 1 to 2 minutes, withdrawn, cooled, and frozen rapidly by immersion in a mixture containing solid carbon dioxide and acetone. They are then rapidly thawed and subjected to a second 2 minute immersion in boiling water. The procedure of freezing and thawing, and heating is repeated, if necessary, until the contents of the tube show intense static double refraction between crossed Polaroid screens. This material will be referred to as the initial gel.

Preparation of Seeding Fibrils—The contents of a vial of initial gel are homogenized by repeated expulsion through a 10 inch, 18 gage hypodermic needle. The homogenate is then frozen in a solid carbon dioxide-acetone bath and rapidly thawed. The freezing-thawing cycle is repeated and the gel is then injected into 10 times its volume of 0.05 N hydrochloric acid. This material is added as rapidly as possible to the solution to be assayed. The freezing and thawing treatment breaks the long fibrils into segments, shortening the reaction time by increasing the number of elongating ends.

Preliminary Experiments

Consideration of Variables—A more detailed consideration of the variables involved in fibril elongation will be given later by one of the authors (D. F. W.). The following were considered to be of importance here: pH, temperature, ionic strength, and the nature of the inorganic anions. These factors will affect the rate at which insulin molecules associate with an individual fibril, and, in conjunction with the total aggregating area (see below) and concentration of free protein, will describe the course of a reaction involving dispersed fibrils. In the assay procedure it is desirable to terminate with the fibrils physically associated in a way which will allow mild centrifugation or filtration to separate them from the suspension medium. To keep the number of available ends maximum they should not form spherites as compact, for example, as those obtained by heating a 1 per cent solution of native insulin in 0.1 N H_2SO_4 (1). Floccules of increasing compactness may be induced by an increase in ionic strength. Some salt also increases the rate of elongation to an extent which justifies an addition of salt before or just after seeding. Chlorides or sulfates of monovalent cations have been added to make the final salt concentration 1 to 3 per cent, the sulfate ion (1) being more effective than the chloride ion. Fibril elongation has been found to have a maximum at pH 1.5 to 2.0. 20 per cent sulfuric acid is therefore added to solutions to be

assayed to bring the pH within this region. Seeded solutions are stirred vigorously, but without frothing, not only to keep the flocs distributed throughout the solution, but to break up the larger flocs, thus reducing the time of diffusion of free insulin to the fibril end. Floc size in a stirred suspension has been found to be 2 to 5 μ.

Quantity of Seeding Material—The over-all growth of a population of fibrils is given by

$$- \frac{dC}{dt} = k \text{ (total aggregating area) } C \qquad (1)$$

where k is the growth constant for unit aggregating area and C is the concentration of free insulin. The aggregating area of a single fibril is a function of its size and shape and is therefore related to the surface area. Although applicable to dispersed systems, Equation 1 cannot be applied easily to the growth of fibrils in spherites and flocs. The effect of varying the number of seeding fibrils may be approximated as follows. If N_1 fibrils, each of constant aggregating area, A, are used in seeding, the total aggregating area is $A \cdot N$. Using Equation 1 and integrating, we obtain

$$N_1 kAt = \ln \frac{C_0}{C} = \ln \frac{1}{\theta} \qquad (2)$$

where θ is the fraction of the original insulin present at time t. If N_2 fibrils had been used, the time necessary to achieve a chosen value of θ would have been changed by a factor N_1/N_2; thus, doubling the number of fibrils is expected to halve the time axis. The approximation represented by Equation 2 has been examined briefly by assaying (biologically) the native insulin present during the course of the reaction (Fig. 1). The linearity of the plots of Fig. 1 indicates that Equation 2 may be applied to seeded solutions of the type described here. Equation 2 therefore suggests that the most effective seeding suspension is one containing the maximum number of fibril segments.

Temperature—Impure solutions impose limitations on the maximum allowable temperature, which will be considered below.

In the preliminary experiments beef still concentrates, clarified by filtration, were acidified by the addition of 20 per cent sulfuric acid to pH 1.5 to 1.6. The concentrates contained sufficient salt. After seeding with a solution prepared as described from beef insulin assaying 23 to 25 units per mg., the concentrates were stirred by a glass rod at 2400 r.p.m. in enclosed vessels at 37.5° (no precipitate in unseeded solutions after several days). The reaction precipitates, obtained by centrifuging at 3200 $\times$ g, were suspended in 10 to 15 times their volume of 1 per cent ammonium chloride at pH 2.0 and centrifuged. This washing cycle was repeated twice additionally. The solutions were then dialyzed against distilled water,

dried at 110° for 12 to 18 hours, and weighed. 2 liter volumes were used and seeded at 20 mg. of fibrils per liter. The solutions were stirred for 28 to 72 hours. It was found that duplicate samples gave recoveries within 3 per cent, that only the seeding fibrils could be recovered on reseeding spent concentrate, and that crystalline insulin added to spent concentrate could be recovered quantitatively on reseeding. When the quantity of seeding material was varied from 20 to 4 mg. per liter of concentrate, the amount of insulin removed was independent of the amount of seed (within 3 per cent), provided sufficient time was allowed for completion of the elonga-
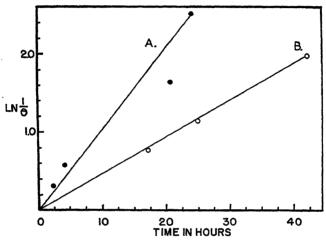
FIG. 1. Fibril precipitation reaction. Line A, beef concentrate (3.09 units per ml.) acidified to pH 1.55. Line B, pork concentrate (2.6 units per ml.) acidified to pH 1.6. Temperature 38°. 2 mg. of seeding fibrils per 100 ml. of concentrate.

tion reaction. The concentrates seeded with 4 mg. per liter were stirred for 75 hours.

On the basis of the above it seemed reasonable to devise an assay procedure to be used with an unselected group of still concentrates. The volumes used in the preliminary assays were considered to be too large for routine use. Choice of a gravimetric analysis suggested still concentrate volumes of 100 ml. (about 20 mg. of insulin). Seeding material, not being critical, was placed at 2 mg. per 100 ml. of concentrate.

Standard Assay

Procedure—The sample to be assayed is filtered with the use of filter aid[1] to a crystal clarity. To 100 ml. of solution in a conical centrifuge tube is

[1] Hyflo Super-Cel, Johns-Manville, New York.

added 1.0 ml. of 20 per cent sulfuric acid (pH 1.5 to 2.0). It is then placed on a stirring apparatus. Seeding fibrils equivalent to 2 mg. of insulin are injected into the sample which is then stirred at 2500 r.p.m. with a straight glass rod for 40 hours at 38° or for 16 to 18 hours at 48°. Care must be taken not to stir air into the solution. The reaction is terminated by centrifuging at 2100 to 2300 $\times$ g until the supernatant is crystal-clear (45 minutes). The fibrous precipitate is transferred to 15 ml. centrifuge tubes with the aid of the first wash solution. Two different procedures for washing the fibrils have been used. In Procedure 1, the precipitate is washed by centrifuging (15 ml. volume) once with 1 per cent ammonium chloride in 0.01 N hydrochloric acid, once with 0.01 N hydrochloric acid, twice with absolute methyl alcohol, and once with absolute ether. In Procedure 2, the precipitate is washed by centrifuging once with 1 per cent ammonium chloride and 0.01 N hydrochloric acid in 65 per cent alcohol, twice with 0.01 N hydrochloric acid in 65 per cent alcohol, and once with reagent grade acetone.

After washing, the wet precipitates are spread over large areas inside the tubes which are wiped with a towel wet with alcohol and dried under a vacuum to constant weight (2 hours or overnight) in the presence of phosphorus pentoxide. The tubes are then washed (inside) with 0.1 N sodium hydroxide, water, alcohol, and ether, dried, and reweighed. The weight of the dried precipitate (less the weight of seeding fibrils) represents the weight of insulin contained in the solution assayed. Transferring and washing the precipitate are facilitated by means of a 5 ml. hypodermic syringe fitted with an 18 gage, 10 inch needle.

Materials—Comparative assays were conducted on the following types of insulin solutions: (*a*) Still concentrates derived from the original acid-aqueous alcoholic extracts of pancreas upon removal of the alcohol by low temperature vacuum distillation. Precipitated lipides were removed by filtration and the resultant product contained 2 to 5 units of insulin per ml., together with some salts and considerable protein material other than insulin. (*b*) Preliminary experiments have been done on solutions of crystalline insulin, 18 to 25 units per mg.; one set of assays was conducted on a crystalline insulin solution subjected to a heat test for stability determination.

Seeding fibrils were prepared from crystalline insulin assaying 25 ± 2 units per mg. by the mouse method.

Results

Table I gives the results of comparative assays on thirty insulin still concentrates. For the fibril assays the precipitation reaction proceeded at 38° for 40 hours.

TABLE I

Comparison of Bioassays and Fibril Assays

Fibril assays were run at 38° for 40 hours.

Sample*	Bioassay		Fibril assay, units per ml.†			Bioassay / Fibril × 100
	Units per ml.	Per cent s.e.	Tube 1	Tube 2	Mean	
B-1	3.6	6.1	3.9	3.9	3.9	92
B-2	3.0	9.2	3.5	3.5	3.5	86
C-3	9.7	9.2	11.7	11.7	11.7	83
B-4	3.9	7.2	3.7	3.5	3.6	108
B-5	3.7	6.4	3.6	3.7	3.6	103
B-6	2.9	9.2	2.9	2.9	2.9	100
P-7	2.6	9.4	3.0	2.9	2.9	90
P-8	3.6	10.3	5.0	4.8	4.9	74
P-9	3.9	8.0	4.1	4.1	4.1	95
P-10	3.7	9.7	4.1	4.1	4.1	90
P-11	3.9	6.9	4.2	4.2	4.2	93
P-12	3.8	9.2	4.0	4.0	4.0	95
P-13	3.6	10.5	4.4	4.6	4.5	80
P-14	3.7	7.2	3.5	3.4	3.4	109
P-15	2.7	8.0	2.3	2.4	2.3	117
P-16	3.4	6.4	3.0	3.1	3.0	113
P-17	2.6	8.3	2.6	2.5	2.5	104
P-18	3.0	9.4	3.0	2.8	2.9	103
P-19	2.6	9.2	3.0	2.9	2.9	90
P-20	2.6	10.7	3.0	3.0	3.0	87
P-21	3.4	9.7	3.1	3.1	3.1	110
P-22	2.6	9.7	2.9	2.9	2.9	90
P-23	3.7	9.0	4.4		4.4	84
P-24	2.9	8.5	3.5	3.3	3.4	85
P-25	3.4	11.5	3.5	3.5	3.5	97
P-26	4.2	8.5	4.2	4.1	4.1	102
P-27	4.0	11.0	4.2	4.2	4.2	95
P-28	3.7	9.2	3.6		3.6	103
P-29	3.5	9.2	4.1	4.3	4.2	83
P-30	3.5	9.2	3.3	3.2	3.2	109
Mean........						95.5

* B, C, and P indicate samples from beef, calf, or pork pancreas.

† The values are derived by multiplying mg. of recovered fibrils by 25/100. This assumes that the fibril precipitate is comparable in purity to insulin containing 25 units per mg.

Table II summarizes the results of similar assays on the thirty-four still concentrates in which the fibril precipitation was obtained by heating at 48° for 16 hours.

Table III gives the results of an experiment on the recovery of a U. S. P.

TABLE II

Biological and Fibril Assays on Still Concentrates

Fibril assays were run at 48° for 16 hours.

Sample*	Bioassay		Fibril assay, units per ml.†			Bioassay / Fibril × 100
	Units per ml.	Per cent s.e.	Tube 1	Tube 2	Mean	
P-31	3.5	7.6	3.5	3.6	3.6	97
P-32	3.4	8.0	4.4	4.4	4.4	77
P-33	2.8	6.7	2.8		2.8	100
P-34	3.2	9.0	3.3	3.4	3.3	97
P-35	3.9	9.2	· 3.4	3.5	3.5	111
P-36	4.2	10.3	4.8		4.8	88
P-37	4.6	9.2	4.2	4.2	4.2	109
P-38	3.4	9.4	3.5	3.5	3.5	97
P-39	4.2	8.5	4.1	4.2	4.1	102
P-40	4.7	8.0	4.7	5.0	4.9	96
P-41	3.0	7.4	2.9	2.9	2.9	103
P-42	4.6	10.6	4.3	4.4	4.3	107
P-43	3.2	9.0	4.1	4.2	4.2	76
P-44	4.2	9.4	4.2	4.2	4.2	100
P-45	2.1	11.7	2.3	2.3	2.3	91
B-46	3.3	9.0	4.3	3.7	4.0	83
B-47	2.6	10.5	2.5		2.5	104
B-48	1.5	9.2	1.5	1.6	1.5	100
B-49‡	3.0	10.3	2.9	2.9	2.9	103
B-50	2.7	6.9	2.4	2.4	2.4	112
B-51	3.1	8.3	3.1	3.0	3.1	100
B-51			2.9	3.0	2.9	
B-51			3.0	3.1	3.1	
B-52	3.4	12.9	4.5	4.2	4.3	79
B-53	4.4	12.9	3.5		3.5	126
B-54	2.1	9.0	2.2	2.2	2.2	96
B-55	4.0	7.6	4.0	4.1	4.1	98
B-56	5.2	9.7	4.7	5.0	4.9	106
C-57	9.9	10.9	11.9	11.9	11.9	83
B-58	2.3	7.4	2.7		2.7	85
B-59	3.4	7.1	2.8		2.8	121
B-60	3.0	11.3	3.1	3.2	3.1	97
B-61	2.7	6.3	2.8	2.9	2.9	93
B-62	5.4	12.0	5.4	5.2	5.3	102
B-63	4.0	11.5	3.7	3.7	3.7	108
B-64	3.0	7.8	3.2	3.2	3.2	94
Mean.........						98.2

* The letters B, C, and P indicate samples from beef, calf, or pork pancreas.
† The values are derived by multiplying mg. of recovered fibril by 25/100.
‡ 65 per cent alcohol washing procedure used in fibril assays on Samples B-49 to B-64. Procedure 1 used in all others.

zinc insulin reference standard from aqueous acid solution, from still concentrate with and without the original insulin removed by fibril precipitation, and from a duplicate assay on the original still concentrate.

The second and third columns in Tables I and II give the bioassay results and their per cent standard errors, while Columns 4 and 5 give the fibril assay results on duplicates in units per ml. of sample, on the assumption that the fibril precipitates obtained contained 25 units per mg. This unit conversion factor of 25 will be considered further in the discussion. Tubes

TABLE III

Recovery of U. S. P. Reference Insulin (23 Units Per Mg.) from Aqueous Solutions and Still Concentrates

Assays were run simultaneously at 48° for 16 hours.

Sample	Tube No.	Insulin added	Units per ml. known	Fibrils recovered	Assayed* units per ml., fibril weight $\times \frac{25}{100}$	Re-covery	Mean re-covery
		mg.		*mg.*		*per cent*	*per cent*
0.01 N HCl in water	1	10.0	2.3	8.7	2.2	96	
	2	10.0	2.3	9.2	2.3	100	
	3	10.0	2.3	9.1	2.3	100	99
Still concentrate, Sample B-51, insulin removed by fibril pre-cipitation	4	10.0	2.3	9.6	2.4	104	
	5	10.0	2.3	9.4	2.4	104	104
Still concentrate, Sample B-51, original sample	6	0	3.0†	11.9	3.0	100	
	7	0	3.0	11.6	2.9	97	
	8	0	3.0	11.9	3.0	100	99
Still concentrate, Sample B-51, original sample	9	10.0	5.3	20.0	5.0	94	
	10	10.0	5.3	20.0	5.0	94	
	11	10.0	5.3	19.2	4.8	91	93

* Procedure 2 (65 per cent alcohol washing) used.

† Previous fibril assay, 3.02 units per ml.; bioassay, 3.1 units per ml. ±8.3 per cent (Table II).

broken from centrifuging, obvious loss of fibrils in withdrawing supernatants, or stirring failure accounts for the lack of some duplicate values. Samples B-49 to B-64 were assayed by Procedure 2 (65 per cent alcohol washing) as described previously. All other results were obtained with Procedure 1.

Pork and Beef Insulin Seeding Fibrils—A number of cross-seeding experiments were performed with beef and pork insulin seeding fibrils on crystalline pork and beef insulin solutions and on pork and beef still concentrates. It appeared from these experiments that pork seeding fibrils enabled the recovery of the full amount of pork or beef insulin from solu-

tion, while beef seeding fibrils gave complete recovery of beef insulin, but only about 50 to 60 per cent recovery of pork insulin. All except Samples 1 to 7 have, therefore, been assayed with pork insulin seeding fibrils. Further examination of these observed differences is in process.

Washing Fibril Precipitate—It is essential to wash the precipitate free of all substances which will interfere with the final measurement (gravimetric in these experiments). Washing by Procedure 1 works well with still concentrates. The fibril precipitate from more purified samples tends to disperse when Procedure 1 is used and cannot be centrifuged easily. Procedure 2, designed to overcome this difficulty, works quite well. Assuming 5 per cent impurity in the residual liquid after the first centrifugation, Procedure 2 should reduce this to about 1 γ. The final acetone wash which facilitates drying may also remove some impurity.

Temperature—With pure insulin solutions the rate of elongation of the fibrils increases by a factor of about 4 for each 10° rise in temperature over a wide range (20–30°; 90–100°). Other factors being constant, a 10° rise in temperature should decrease the assay time by the same factor. When pure insulin is to be converted to fibrils, relatively high temperatures may be used (*e.g.*, 100°). However, in assaying, no precipitate other than insulin should form in seeded samples or unseeded controls. For still concentrates we have chosen temperatures (48° or below) at which the unseeded controls remain clear. A precipitate forms increasingly at temperatures of 52° or above. The overnight reaction time at 48° is considered practical, for a minimum of working hours is involved.

It is possible that higher temperatures may be used, particularly for the assay of more purified samples. This matter is under investigation.

DISCUSSION

The precision of the fibril assay procedure, independent of the assumed conversion factor of 25 units per mg., appears to be within ±5 per cent as indicated by the variance of duplicates. The third column of Tables I and II indicates standard errors of 6 to 13 per cent for the bioassay used. A measured deviation from actual insulin content of 2.6 standard errors could be expected once in 100 trials on the basis of statistical variation alone. In addition, the interference of impurities on absorption in mice and the possibility of incomplete precipitation in preparing extracts for bioassay constitute other sources of error.

In the last column of Tables I and II the bioassays are expressed as per cent of the fibril assays. Good agreement is obtained within the limits of the bioassay error. The greatest differences are for Samples P-32 and B-59 in which deviations of 2.9 and 3.0 standard errors are obtained. A further analysis of the limits of the fibril assay will require more accurate

bioassays and a thorough analysis of the regenerated insulin obtained from the assay precipitates. The reliability of the precision accuracy indicated above (5 per cent) is strengthened, however, by the recoveries of crystalline insulin from pure solution and after addition to normal and spent still concentrates as shown in the preliminary experiments and in Table III.

We have assumed that the weight-potency conversion factor (25 units per mg.) of a good grade of crystalline insulin applies to the fibril precipitates. That this value is reasonable may be deduced from the following. First, the assays of Tables I and II have been compared by use of a value of 25 units per mg. The ratios of the assays have a relatively normal distribution about the mean. The fact that the fibril assay indicates about 2 to 5 per cent more insulin than the bioassay does not affect this interpretation. Second, the results shown in Table III for the assay procedure indicate a quantitative recovery of crystalline insulin from pure solution and after addition to normal and spent concentrates. Third, two samples of purified insulin containing 18.3 and 28.7 units per mg. (± 6.5 per cent) by bioassay gave 16.9 and 24.8 units per mg. by fibril assay. Fourth, the fibril precipitates obtained with the technique described may be made to revert to a product having an activity close to 25 units per mg. Some forms of biologically inactive insulin may be found which will interfere, in the sense that they contribute to the fibril precipitate (but not to activity). This possibility is being examined. All of the experiments thus far, however, suggest that the technique reported here will be useful in research problems as well as in those associated with the production and purification of the hormone.

The fibril assay has also been used to follow the results of the standard heat test on one sample of insulin (40 units per ml., pH 3.0, 52°, and 10 days). Bioassay of the control and heat-tested solutions gave 38.4 units per ml. ± 14 per cent and 27.3 units per ml. ± 7.1 per cent, respectively. Fibril assays resulted in values of 38.3 and 27.5 units per ml. with or without filtration of the original samples. The latter indicates that the biologically inactive insulin produced during the course of the test is not measured in the fibril assay; therefore, the loss is probably not due to fibril formation in this experiment.

SUMMARY

1. A method for the assay of insulin *in vitro*, based on the specific elongation reaction of the insulin fibril, was devised. The over-all process is termed fibril precipitation.

2. The method *in vitro* was compared with a biological mouse convulsion method on 64 unselected crude pancreas extracts (still concentrates) and several solutions of crystalline insulin. The fibril assay described has a

precision error within ±5 per cent.   Assuming the fibril assay to be correct, the bioassays had a distribution over a range of 74 to 126 per cent of the fibril assay values.   This range is reasonable on the basis of random selection of mice alone.

3. It was found that the fibril precipitation assay could be conducted at 38° for 40 hours or at 48° for 16 hours (overnight).   Two other washing procedures were examined.   One, involving 65 per cent alcohol, was found most practical from the standpoint of separation of the precipitate by centrifugation.

4. An apparent difference between pork and beef insulin seeding fibrils was found, pork fibrils being the most effective for seeding purposes.

5. Two samples of insulin having 18.3 and 28.7 units per mg. by bioassay gave 16.9 and 24.8 units per mg. by fibril assay.

6. The biologically active insulin remaining after a standard heat test applied to one sample was measured accurately by the fibril assay.

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STUDIES ON NATURAL AND RACEMIC AMINO ACIDS WITH RATS*

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We have previously reported that glycine, DL-phenylalanine, and L-proline inhibit the growth of young rats when added to an adequate diet at a 5 per cent level (1). Toxicity of amino acids has been reported by other workers (2–6). We have extended these studies to a number of other amino acids and have shown differences, in some cases, between the growth-inhibiting effects of natural isomers and racemic mixtures. In the case of aspartic acid a more thorough study has been made.

EXPERIMENTAL

In the preliminary experiments 21 day-old male albino rats weighing 40 ± 2 gm. were divided into groups of five each and fed the experimental diets *ad libitum*. The diets contained 20 per cent Labco casein and other ingredients, as previously reported (1). The amino acids were fed at a 2 or 5 per cent level of the diet.

Subsequently, the effects of feeding L- and DL-aspartic acids at a 5 per cent level were similarly studied on groups of ten rats each. After 2 weeks on this diet, the animals were kept in metabolism cages for 5 days and the urine collected under toluene. No food was offered in these cages, but the rats were transferred to other cages for feeding each morning and afternoon for 2 hour periods. We were thus able to collect uniform urine samples which were not contaminated with food or amino acids. These samples were analyzed for "free" and total amino acids by the method of Woodson *et al.* (7).

Results

Table I shows the average weight gain per rat for a 3 week period. Addition of L-leucine to the diet caused no inhibition of growth, whereas DL-leucine caused a slight inhibition at the 5 per cent level. Glutamic acid or lysine had no inhibitory effect, regardless of the form fed. L-Tryptophan inhibited growth slightly, while DL-tryptophan was more severe

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in effect. The DL form of aspartic acid was inhibitory, while the L form was not. Addition of 5 per cent L-tyrosine to the basal diet did not inhibit growth but caused large amounts of tyrosine to appear in the urine.[1] This urine appeared dark brown in color, indicating a state of alkaptonuria such as reported by Schweizer (8).

Table II shows the urinary content of "free" and total amino acids found for rats fed the basal diet and those fed the basal diet supplemented with 5 per cent L- or DL-aspartic acid. No significant differences from the controls are apparent, except for aspartic and glutamic acid excre-

TABLE I

Effect of Feeding L- and DL-Amino Acids on 21 Day Weight Gain of Rats

The basal diet which contained no added amino acids allowed an average gain per rat of 82 gm.

Amino acid*	L isomer		DL mixture	
	Per cent added	Average gain per rat	Per cent added	Average gain per rat
		gm.		*gm.*
Leucine........................	2	78.0	2	75.5
	5	86.0	5	63.0
Glutamic acid.................	2	78.0	2	82.0
	5	82.0	5	75.5
Lysine·HCl....................	5	78.0	5	78.0
Tryptophan...................	2	63.0	2	52.5
	5	42.0	5	35.6
Aspartic acid..................	2	78.0	2	84.0
	5	71.0	5	46.0
Tyrosine......................	5	76.0†		

* Merck.

† Analysis of the urine from this group for "free" and total amino acids showed no significant differences from the urine of rats on the basal diet, except for tyrosine. Excretion of this amino acid per rat per day was as follows: On basal diet, 110 γ (free); 350 γ (total). With added tyrosine, 3100 γ (free); 3650 γ (total)

tion. The rats fed DL-aspartic acid excreted 50 times more total aspartic acid and 20 times more total glutamic acid than the control group. The rats fed L-aspartic acid showed no deviation from normal.

α-Amino nitrogen was determined in these urines by the HNO_2 method of Peters and Van Slyke (9) and the copper method of Pope and Stevens (10) as employed by Albanese and Irby (11), and the results recorded in Table III. Values obtained by the Van Slyke procedure are much

[1] In previous tests not shown in Table I, DL-valine, DL-threonine, L-arginine hydrochloride, DL-isoleucine, and DL-serine at a 5 per cent level in the diet did not inhibit growth. DL-Methionine was markedly inhibitory and L-cystine caused death in 6 to 8 days at this level.

higher than those obtained by the copper method. The former procedure is known to respond to substances other than amino acids (12). This discrepancy between the amounts of α-amino nitrogen as determined by these two methods has been reported by Sauberlich and Baumann (13). The differences in our experiments are much greater than those reported by these authors.

TABLE II

Effect of Feeding L- and DL-Aspartic Acids on Urinary Excretion of "Free" and Total Amino Acids by Rats

Urine was collected for 20 hours per day for 5 days. The values are reported in mg. per gm. of urine solids. At the end of the first 2 weeks the control rats averaged 108 gm. and those receiving L-aspartic acid 106 gm. The rats receiving DL-aspartic acid averaged 75 gm.

Amino acid	Supplement to basal diet					
	None		5 per cent L-aspartic acid		5 per cent DL-aspartic acid	
	Free	Total	Free	Total	Free	Total
Arginine	0.48	0.64	0.43	0.56	0.35	0.43
Aspartic acid	0.24	2.42	0.09	2.18	101.00	104.00
Glutamic "	0.68	2.54	0.85	1.80	35.50	36.80
Histidine	0.09	0.27	0.09	0.22	0.16	0.26
Isoleucine	0.20	0.95	0.22	0.87	0.11	0.45
Leucine	0.05	0.77	0.04	0.64	0.05	0.53
Lysine	0.22	1.52	0.04	1.09	0.34	1.28
Methionine	0.26	0.32	0.26	0.28	0.16	0.24
Phenylalanine	0.18	1.04	0.03	0.87	0.17	0.74
Proline	0.65	1.95	0.54	2.00	0.54	2.30
Threonine	0.41	1.28	0.33	1.16	0.30	0.86
Tryptophan	0.31	0.33	0.25	0.25	0.19	0.20
Tyrosine	0.16	0.51	0.05	0.44	0.15	0.43
Valine	0.05	0.74	0.04	0.74	0.05	0.47
Total volume, *cc*	165.0		198.0		218.0	
" solids, *gm*	8.55		11.0		10.2	

Although the results obtained by the two methods did not agree, comparable results were obtained on the urines from rats on the basal diet and from rats on the basal diet supplemented with L-aspartic acid when the same method was employed. Thus, these two urines appear to be similar according to these two chemical assays as well as microbiologically, as was shown in Table II. The higher values obtained on the unhydrolyzed urine of rats which received DL-aspartic acid are probably due to the presence of D-aspartic acid in the urine.

Both of these methods yield higher values for α-amino nitrogen than

those calculated from microbiological determinations of the amino acids.[2]
This is in agreement with Sauberlich and Baumann (13), who have thoroughly discussed the possibilities for this discrepancy.

Heparinized blood samples from animals in each group were pooled and
the proteins precipitated from the plasma as described by Hier and Bergeim (14). These were analyzed for aspartic and glutamic acids and the
results expressed in Table IV.

TABLE III

α-Amino Nitrogen Excreted by Rats after Feeding L- and DL-Aspartic Acids

The values are reported in mg. of α-amino nitrogen per gm. of urine solids before and after hydrolysis.

Method	Supplement to basal diet					
	None		5 per cent L-aspartic acid		5 per cent DL-aspartic acid	
	Before	After	Before	After	Before	After
Van Slyke HNO$_2$............	24.4	91.5	26.5	99.5	39.3	88.2
Copper......................	4.25	40.5	7.0	40.3	23.3	56.0

TABLE IV

Effect of Feeding L- and DL-Aspartic Acids on "Free" Aspartic and Glutamic Acid Level of Rat Plasma

The values are reported as micrograms per cc. of plasma.

Amino acid	Supplement to basal diet		
	None	5 per cent L-aspartic acid	5 per cent DL-aspartic acid
Aspartic acid............	0	0	109
Glutamic "	45	37	47

The amount of aspartic acid found in the plasma from normal rats and
those receiving L-aspartic acid was nil. This agrees with the results reported by Henderson *et al.* (15). However, an appreciable amount of
aspartic acid was found in the plasma of rats receiving DL-aspartic acid in
the food. Christensen *et al.* (16) have reported that the feeding of L-aspartic acid does not elevate the plasma aspartic acid, but these workers
did not feed the DL mixture.

The quantity of glutamic acid present in these plasma samples was
not influenced by the presence of L- or DL-aspartic acid in the food.

[2] This is also true for human urine. Unpublished data.

DISCUSSION

As we have previously reported (17), the DL mixtures of some amino acids are more inhibitory to growth than the natural isomers. This emphasizes the importance of the correct balance of amino acids in the diet of the rat and suggests caution in the use of large doses of certain amino acids, particularly the unnatural forms or racemic mixtures. The greater degree of inhibition caused by the racemic forms is in agreement with the observation of Howe et al. (18) that racemic mixtures of amino acids are less well tolerated than the natural forms when given intravenously.

Although Sullivan et al. (2), Martin (5), and Schweizer (8) have reported L-tyrosine to be toxic to the rat in high levels, we have not found this under the conditions of our experiments. Neither have we produced various pathological lesions such as those reported by these authors. These differences in results between laboratories are probably due to a difference in basal diets.

We did not determine the proportions of D- and L-aspartic acids present in the plasma and urine in these experiments since the organism employed (*Leuconostoc mesenteroides* P-60) utilizes both forms. However, the rats fed L-aspartic acid showed no increase in blood or urinary level of this amino acid, while the rats fed DL-aspartic acid showed an increase. It is probable, therefore, that the increase was due to unmetabolized D-aspartic acid.

It is difficult to explain the fact that the urinary level of glutamic acid was higher in the animals fed DL-aspartic acid than in that of the other groups, despite the fact that there was no increase in the plasma level. It is possible that the glutamic acid exists in a microbiologically unavailable form in the plasma.

The authors wish to express their appreciation to Dr. David Klein for his interest and support and to Donald E. Ryan, Theodora Jaksibaga, and Phyllis Overling for technical assistance.

SUMMARY

Rats have been fed 2 or 5 per cent of various amino acids in the natural or racemic form, replacing an equivalent amount of sucrose in a complete casein diet.

The DL mixtures of leucine, tryptophan, and aspartic acid prevented growth to a greater extent than the natural isomers. The possible significance of these findings is discussed.

The amino acid pattern of urine from rats on the basal diet has been

compared with that from rats receiving L- or DL-aspartic acid in the diet and some differences noted.

Chemical determinations of α-amino nitrogen in these urines by two methods have yielded different values, both of which are significantly higher than can be accounted for microbiologically.

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THE LOCATION IN PROTOPORPHYRIN OF THE CARBON ATOMS DERIVED FROM THE α-CARBON ATOM OF GLYCINE*

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Previous studies on the biosynthesis of protoporphyrin, the porphyrin moiety of hemoglobin, have demonstrated not only that glycine can serve as the nitrogenous precursor of the porphyrin (1, 2), but also that glycine nitrogen is the precursor of both types of pyrrole rings of protoporphyrin (*i.e.* rings *A* and *B* and *C* and *D*) (3–5). Also it was shown that, while the α-carbon atom of glycine is utilized for porphyrin synthesis (6, 7), the carboxyl carbon atom of glycine does not enter the porphyrin molecule (7, 8). On further investigation it was found that not only is the α-carbon atom of glycine utilized but that for every 4 nitrogen atoms utilized, 8 α-carbon atoms of glycine apparently enter the porphyrin molecule (7).

This paper presents degradation experiments undertaken to locate the positions of the carbon atoms in protoporphyrin which are derived from the α-carbon atom of glycine. C^{14}-labeled porphyrin was obtained by incubating C^{14}-methylene-labeled glycine ($C^{14}H_2NH_2COOH$) in duck blood (9). The labeled heme formed was degraded according to the outline described in Fig. 1. The hemin was converted to the iron-free protoporphyrin, which in turn was reduced to mesoporphyrin. The mesoporphyrin was oxidized to yield methylethylmaleimide (from pyrrole rings *A* and *B*) and methyl-β-carboxyethylmaleimide or hematinic acid (from pyrrole rings *C* and *D*). The hematinic acid was decarboxylated to yield methylethylmaleimide. The samples of methylethylmaleimide (from pyrrole rings *A* and *B* and *C* and *D*) were further degraded separately. They were oxidized to their glycols (substituted tartarimides) and the 2-(3)-methyl-3-(2)-ethyltartarimides were further oxidized to yield pyruvic and α-ketobutyric acids. Only the α-ketobutryric acid was radioactive. Both

the free α-ketobutyric acid and its 2,4-dinitrophenylhydrazone derivative were decarboxylated to yield propionic acid and CO_2. Of these only the CO_2 was radioactive. In the degradation scheme in Fig. 1, the carbon

HEMIN→ PROTOPORPHYRIN IX → MESOPORPHYRIN →

METHYLETHYLMALEIMIDE

→ HEMATINIC ACID $\xrightarrow{CO_2 (C10, D10)}$ + METHYLETHYLMALEIMIDE →

2-(3)-METHYL-3-(2)ETHYLTARTARIMIDE

CH_3——CO——COOH
$(A6, B6$—$A4, B4$—$A5, B5)$
+
CH_3——CH_2——CO——COOH
$(A9, B9$—$A8, B8$——$A3, B3$—$A2, B2)$

$\rightarrow$

CH_3—CH_2—COOH
$(A9, B9$-$A8, B8$-$A3, B3)$
+
CO_2
$(A2, B2)$

CH_3——CO——COOH
+
CH_3——CH_2——CO——COOH
$(C9, D9$—$C8, D8$——$C3, D3$—$C2, D2)$

$\rightarrow$

CH_3—CH_2—COOH
$(C9, D9$-$C8, D8$-$C3, D3)$
+
CO_2
$(C2, D2)$

FIG. 1. Outline of hemin degradation. The letters and numbers underneath the carbon atoms designate the positions of these atoms in protoporphyrin.

atoms of the fragments isolated are numbered according to their positions in the protoporphyrin from which they are derived (see below).

Analysis of the C^{14} activities of the various compounds enables one to locate the radioactive carbon atoms of the protoporphyrin (Table I). In

the pyrrole units, only carbon atoms in positions $A2$, $B2$, $C2$, and $D2$ (see below) were radioactive, and since they contained but 50 per cent of the total activity, the methene carbon atoms must also be derived from the α-carbon atom of glycine. The carbon atoms of the protoporphyrin derived from the α-carbon atom of glycine are shown in Fig. 2.

In this and forthcoming studies on the biosynthesis of porphyrins, certain predetermined carbon atoms of the porphyrin molecule are isolated by suitable degradation procedures. The numbering system used by Fischer is inadequate to describe clearly the location in the porphyrin of an individual carbon atom isolated in the degradation experiments. It is

PROTOPORPHYRIN   9

$\overset{\bullet}{C}H_2NH_2COOH$

FIG. 2. Positions in protoporphyrin derived from the α-carbon atom of glycine

therefore desirable to introduce a numbering system for porphyrins which can designate a particular carbon atom without ambiguity.
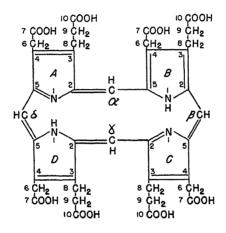
The proposed system is based partially on Fischer's. The rings are designated with capital letters A, B, C, D, corresponding to Fischer's I, II, III, and IV respectively; the methene carbon atom bridges are given Greek letters α, β, γ, and δ, as in Fischer's system, and the remaining atoms are numbered. The ring carbon atoms are numbered in such a fashion that similar side chains occur on the same numbered β-carbon atom of each pyrrole ring. Therefore, in the porphyrin III series, ring D of necessity is numbered clockwise instead of counter-clockwise as in rings A, B, and C. However, in the porphyrin I series all four rings will be numbered counter-clockwise.

Since uroporphyrin, a C_{40}-porphyrin, is the biological porphyrin contain-

ing the largest number of carbon atoms, it is used as the parent compound (see Fig. 3). The carbon atoms of protoporphyrin, the porphyrin found in hemoglobin, would therefore be numbered as shown in Fig. 1.

With this nomenclature for porphyrins, it is comparatively easy to designate a particular carbon atom; *i.e.*, carbon atom $A4$ refers to the β-carbon atom of pyrrole ring A of protoporphyrin to which the methyl group is attached. Carbon atoms $A5$, $B5$, $C5$, and $D5$ are the α-carbon atoms of the same side as the methyl side chains of pyrrole rings A, B, C, and D respectively.

This system of numbering the atoms of prophyrin can also be made to apply to the plant porphyrins, the chlorophylls.

UROPORPHYRIN III

FIG. 3

EXPERIMENTAL

Measurement of Radioactivity—All the samples were assayed for their radioactivity as barium carbonate. The organic compounds were burned and the carbon dioxide formed was collected as barium carbonate, which was deposited as an "infinitely thick" layer on a standard dish. The C^{14} values of each sample are expressed as counts above background per minute per standard dish for barium carbonate at infinite thickness. Sufficient counts were usually taken to reduce the statistical counting error to about 2 per cent; the error of the measurements was within 5 per cent.

For convenience in comparing the isotope concentrations of different compounds the radioactivities are also given as *total activity*. Total activity is defined as the value obtained by multiplying the activity found

by the number of carbon atoms in the compound analyzed. Total activity is proportional to the activity per mole of compound.

Methylene-Labeled Glycine $(C^{14}H_2—NH_2—COOH)$—The glycine was prepared from methyl-labeled sodium acetate obtained from Tracerlab (lot No. 10) by the method of Radin and Engelman as described by Radin *et al.* (7).

TABLE I

Distribution of C^{14} in Protoporphyrin after Incubation of Duck Blood with N^{15}-Labeled and Methylene-Labeled Glycine $(N^{15}H_2—C^{14}H_2—COOH)$

In experiment 1 the porphyrin had a found C^{14} activity of 154 c.p.m., a total activity of 5236 (154 × 34), and an N^{15} concentration of 0.078 atom per cent excess N^{15}.

Compound isolated	Positions of carbon atoms in porphyrin	Pyrrole rings A and B		Pyrrole rings C and D	
		Activity found	Total activity	Activity found	Total activity
		c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*
Hematinic acid	Rings C and D			85	680
Methylethylmaleimide	" A and B; C and D	94	660	92	644
Pyruvic acid	CH_3——CO——$COOH$ $A6, B6$ $A4, B4$ $A5, B5$ $C6, D6$ $C4, D4$ $C5, D5$	0	0	0	0
2,4-Dinitrophenylhydrazone of α-ketobutyric acid	CH_3——CH_2——CO——$COOH$ $A9, B9$, $A8, B8$ $A3, B3$ $A2, B2$ $C9, D9$, $C8, D8$ $C3, D3$ $C2, D2$	63	630	66	660
COOH group of α-ketobutyric acid (from hydrazone)	$A2, B2$ $C2, D2$	100	700	116	812
CO$_2$ from hematinic acid	—$COOH$ $C10, D10$			4	4
Propionic acid	CH_3——CH_2——$COOH$ $A9, B9$ $A8, B8$ $A3, B3$ $C9, D9$ $C8, D8$ $C3, D3$	0	0	1	3

The yield of glycine was 77 per cent. The glycine had an activity of 0.1 mc. per mm and gave 282,000 c.p.m. as measured by the above method.

$C_2H_5O_2N$. Calculated, C 32.0, H 6.7; found, C 32.0, H 6.7

This C^{14}-labeled glycine was then mixed with an equal amount of glycine labeled with 32.4 atom per cent excess N^{15}. Therefore the glycine used had a C^{14} activity of 141,000 c.p.m. and contained 16.2 atom per cent excess N^{15}. This sample was used in Experiment 1. The glycine used in Experiment 2 had an activity of 126,000 c.p.m. and contained 16 atom per cent excess N^{15}.

The experimental procedures for the preparation and degradation of the hemin are described below for Experiment 1. The procedures used in Experiment 2 were the same.

Preparation of Labeled Hemin from N^{15}-Labeled and α-Carbon-C^{14}-Labeled Glycine ($N^{15}H_2$—$C^{14}H_2$—COOH)—Heparinized blood (1095 cc.) from ten ducks was incubated with the doubly labeled glycine for 24 hours at 37° in a manner previously described (9). The blood of each duck was incubated separately with 1 mg. of the glycine per cc. The hemin was isolated in the usual manner; the activity was 226 c.p.m.[1] and the N^{15} concentration 0.110 atom per cent excess.

In order to have enough material for the degradation studies, 2.255 gm. of the labeled hemin were diluted with 1.007 gm. of non-isotopic hemin.

Preparation of Protoporphyrin—The hemin was converted to protoporphyrin by treatment with powdered iron in boiling formic acid (10). The product, which weighed 2.69 gm. (96 per cent yield), was used without purification. The absorption spectrum indicated that it was contaminated with some mesoporphyrin.

Preparation of Mesoporphyrin—The protoporphyrin was dissolved in 150 cc. of 100 per cent formic acid and hydrogenated at room temperature and at atmospheric pressure with the aid of a colloidal palladium catalyst (11). The mixture was filtered and poured into 3 volumes of 30 per cent ammonium acetate to flocculate the mesoporphyrin. If the precipitation was incomplete, the filtrate was extracted with ethyl acetate and the material from the extraction was added to the bulk of the product. The washed mesoporphyrin was dissolved in 2 per cent ammonium hydroxide and reprecipitated by the addition of acetic acid. The collected precipitate was washed and further purified by dissolving it twice more in ammonium hydroxide and precipitating it with acetic acid. The yield was 2.54 gm. or 94 per cent.

Oxidation of Mesoporphyrin to Methylethylmaleimide and Hematinic Acid —A solution of 5.1 gm. of chromic acid in water was added with stirring over a period of several hours to a solution of 2.52 gm. of mesoporphyrin in 170 cc. of 20 per cent sulfuric acid (12, 13). Stirring was continued overnight and the methylethylmaleimide was separated from the hematinic acid as described previously (4).

The isolated methylethylmaleimide was purified by repeated sublimation. The yield was 0.53 gm. (42 per cent); m.p. 63.5–65° (Küster (12) reported 67°; Fischer *et al.* (13) reported 70°).

$C_7H_9O_2N$.   Calculated, C 60.4, H 6.5; found, C 60.4, H 6.3

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[1] Determined as hemin and calculated as barium carbonate.

The hematinic acid was isolated and purified as previously described (4). The yield was 0.65 gm. (40 per cent); m.p. 114.4–115.1° (Küster (14) reported 113.5–114.5°).

$C_8H_9O_4N$. Calculated, C 52.5, H 4.9; found, C 52.5, H 4.9

Conversion of Hematinic Acid to CO_2 and Methylethylmaleimide—This conversion is a base-catalyzed decarboxylation essentially as described by Küster (14, 15). A suspension of 0.64 gm. of hematinic acid in 10 cc. of a nearly saturated ammoniacal solution of absolute ethanol was heated in a sealed tube for 2 hours at 175°. After this time the reaction mixture was diluted with water and acidified with sulfuric acid, and the formed carbon dioxide was aerated into barium hydroxide. The yield of barium carbonate was 0.50 gm. (73 per cent).

After the aeration process the solution was made alkaline with dilute sodium bicarbonate and immediately extracted six times with ether. The ether solution was evaporated, the residue dissolved in acidulated water, and the product purified by steam distillation. The distillate was made slightly acid with sulfuric acid and extracted with ether in a continuous extractor. The ether solution was dried over drierite and evaporated, and the product purified by sublimation. The yield was 0.15 gm. (30 per cent); m.p. 61–63°.

$C_7H_9O_2N$. Calculated, C 60.4, H 6.5; found, C 61.1, H 6.2

Preparation of 2-(3)-Methyl-3-(2)-ethyltartarimide—The methylethylmaleimide was oxidized with sodium chlorate and osmium tetraoxide in aqueous solution by the method of Milas and Terry (16). Maximum yields were obtained by carrying out the reaction at room temperature for about 20 hours. Titration of an aliquot of the reaction mixture with periodic acid indicated that the oxidation was quantitative.

Preparation of Pyruvic and α-Ketobutyric Acids—The tartarimide was cleaved to pyruvic and α-ketobutryic acids by means of periodate (17). An exact equivalent of sodium metaperiodate was added to the above aqueous solution of the tartarimide. After standing for 20 minutes, the solution was extracted with ether for 5 hours in a continuous extractor. The ether was taken to dryness and the residue dissolved in 1 cc. of 0.5 N HCl and heated for 1 hour at 80°. The solution was then mixed with 1 gm. of anhydrous silica gel and transferred, with the aid of a small volume of butanol-chloroform solution, to a chromatographic column for separation of the acids.

Separation of Pyruvic and α-Ketobutyric Acids—The pyruvic and α-ketobutyric acids were separated by partition chromatography on silica gel according to Isherwood's method (18). A column 1.6 cm. in diameter

and 21 cm. in length containing 10 gm. of silica gel was used for 50 to 200 mg. quantities.   The stationary phase was 0.5 N sulfuric acid, and the moving phase was a 7 per cent butanol-chloroform solution saturated with 0.5 N sulfuric acid.   The rate of flow through the column, 0.7 cc. per minute, was adjusted by the head of pressure.

The effluent from the column was collected in 5 cc. fractions, and each fraction was slurried with water and titrated with 0.01 N sodium hydroxide to the end-point of brom thymol blue.   The chromatograms are
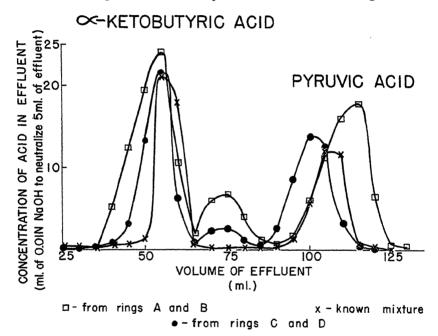
Fig. 4. Chromatographic separation of pyruvic and α-ketobutyric acids

shown in Fig. 4.   A small amount of acid of unknown composition emerged between the two main peaks; this was not identified.   The separations were reproducible, and the positions of the peaks serve as a criterion of identification.

The keto acids were isolated as their 2,4-dinitrophenylhydrazones. The aqueous phases from the titrated effluent, containing the sodium salts, were separated, concentrated to a small volume, and acidified with hydrochloric acid.   The indicator was removed with Darco G-60, and an equivalent amount of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid was added.   The yields of the acids were 30 to 50 per cent, based on

the methylethylmaleimide. The hydrazone of the pyruvic acid was recrystallized from ethanol; the hydrazone of the α-ketobutyric acid was recrystallized by the addition of water to a hot glacial acetic acid solution.

The pyruvic acid hydrazones from pyrrole rings A and B and C and D melted at 218–220° and 217–218°, respectively, and gave no depression in melting point when mixed with an authentic sample, m.p. 218–220°.

The α-ketobutyric acid hydrazone from pyrrole rings A and B and C and D melted at 197° and 196–198°, respectively, and gave no depression in melting point when mixed with an authentic sample,[2] m.p. 200°.

2,4-Dinitrophenylhydrazone of pyruvic acid
$C_9H_8O_6N_4$.   Calculated.   C 40.3, H 3.0, N 20.9
From pyrrole rings A and B.   Found.   " 40.3, " 2.8, " 20.6
"   "   " C and D.   "   " 40.3, " 3.2, " 20.3
2,4-Dinitrophenylhydrazone of α-ketobutyric acid
$C_{10}H_{10}O_6N_4$.   Calculated.   C 42.6, H 3.6, N 19.9
From pyrrole rings A and B.   Found.   " 43.4, " 3.8, " 20.7
"   "   " C and D.   "   " 42.5, " 3.8, " 20.2

Decarboxylation of α-Ketobutyric Acid—The dinitrophenylhydrazone of α-ketobutyric acid was oxidized according to the method of Krebs (19) with an excess of potassium permanganate solution containing 5 per cent sulfuric acid, and the carbon dioxide formed was aerated into barium hydroxide. Theoretically, 7 moles of carbon dioxide are formed per mole of hydrazone. Since we have found that the yield of propionic acid was higher than the yield of CO_2, it would appear that the oxidation of the benzene ring was less complete than the decarboxylation. Therefore the activity of the isolated CO_2 was slightly higher than theory. However, the CO_2 was radioactive and the propionic acid was devoid of C^{14} activity.

The excess permanganate was then reduced with oxalic acid and the propionic acid was steam-distilled. The distillate was titrated with 0.01 N NaOH and concentrated to 2 to 4 cc. The propionic acid was converted to its silver salt with silver nitrate. This sample was used for the C^{14} determination. It was subsequently converted to its sodium salt.

$C_3H_5O_2Na$.   Calculated.   C 37.5, H 5.3, Na 23.9
            Found.   " 36.0, " 4.8, " 24.6

That the activity of the α-ketobutyric acid resides only in the carboxyl group was confirmed by decarboxylation of a crude sample of sodium α-ketobutyrate, obtained from the column, with ceric sulfate (20). The CO_2 had an activity of 546 c.p.m., while the propionic acid, isolated as its silver salt, was inactive.

[2] We are indebted to Dr. D. B. Sprinson for a sample of α-ketobutyric acid.

The methylethylmaleimide (from pyrrole rings A and B) and hematinic acid (from pyrrole rings C and D), which were isolated, represent 30 carbon atoms of the total 34 carbon atoms of the protoporphyrin, and, as will be shown, account for 50 per cent of the total radioactivity of the porphyrin. The radioactivity of these compounds is due to the activity of 4 carbon atoms in positions $A2$, $B2$, $C2$, and $D2$ since neither the pyruvic acid nor the propionic acid from the α-ketobutyric acid nor the carboxyl group of hematinic acid contained any activity (Table I). The active carbon atoms $A2$, $B2$, $C2$, and $D2$ were isolated from the carboxyl group of the α-ketobutyric acid fractions. Therefore out of the 30 carbon atoms examined 4 were derived from the α-carbon atom of glycine. The average activity of these 4 carbon atoms is best calculated from the activities of the methylethylmaleimides, the hematinic acid, and from the hydrazones of the α-ketobutyric acids. (The direct determination of the activity of the carboxyl group is not accurate for reasons discussed in the experimental section.) Since only 1 carbon atom of each of these compounds is active, its activity is equal to the total activity of the compound (Table I); *i.e.*, the found activity of the compound analyzed multiplied by the number of carbon atoms in the particular compound. The average value of this carbon atom is 645 c.p.m. for pyrrole rings A and B and 662 c.p.m. for pyrrole rings C and D. The average activity of these 4 radioactive carbon atoms is 654 c.p.m. and their total activity is 2616 (4 × 654). The total activity of the porphyrin is 5236 (34 × 154).

The activities of carbon atoms in positions $A2$ and $B2$ are equal to the activities of carbon atoms in positions $C2$ and $D2$, and it is evident that the α-carbon atom of glycine is utilized equally for the biosynthesis of both types of pyrroles in the protoporphyrin molecule. The utilization of N^{15}-labeled glycine has also been found to be the same for both types of pyrrole rings (4). It can readily be seen that the 4 active carbon atoms occupy comparable positions in both types of pyrrole rings. This finding, taken together with the finding that the activities of both types of pyrrole rings are equal, is in harmony with the hypothesis suggested by the N^{15} utilization (4) that in the biosynthesis of protoporphyrin a pyrrole ring is formed which is a common precursor of both types of pyrrole structures found in the protoporphyrin.

It may be well to point out that in the present degradation experiments the pyrrole rings were isolated as pairs, pyrrole rings A and B and C and D. It is of course possible but highly improbable that only 1 carbon atom of each pair is derived from the α-carbon atom of glycine.

Since the total activity of the porphyrin was 5236, there are 2620 (5236 − 2616) total counts unaccounted for. This activity must

reside in the carbon atoms of the methene bridges, since these 4 carbon atoms are the only ones which were not examined. The same conclusion was arrived at from a similar experiment whose data are given in Table II. A summary of these calculations is given in Table III.

From the radioactivity of these carbon atoms the number of the methene carbon atoms derived from the α-carbon atom of glycine may be

TABLE II

Distribution of C^{14} in Protoporphyrin after Incubating Duck Blood with N^{15}-Labeled and Methylene-Labeled Glycine (N^{15}—H_2—$C^{14}H_2$—$COOH$)

In experiment 2 the labeled hemin isolated was diluted 4-fold with non-isotopic hemin before counting and degradation. The N^{15} concentration was 0.043 atom per cent excess before dilution.

Compound	Found activity	Total activity
	c.p.m.	*c.p.m.*
Mesoporphyrin..........................	44.2	1503
Methylethylmaleimide (pyrrole rings A and B).................................	25.7	180
Hematinic acid (pyrrole rings C and D).....	23.9	191

TABLE III

C^{14} Activity of Fragments of Porphyrin Molecule
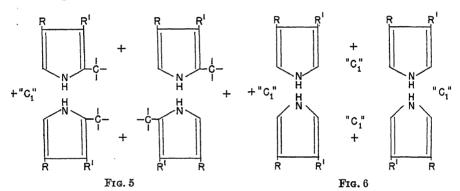
Porphyrin fragment	Total activity	
	Experiment 1	Experiment 2
	c.p.m.	*c.p.m.*
Porphyrin................................	5236	1503
Pyrrole rings A and B.....................	1290	360
" " C and D.....................	1324	382
" " A and B, C and D............	2614	742
4 methene carbon atoms...................	2614*	761*

* Calculated (see the "Discussion").

deduced. The average activity of the methene carbon atoms is 655 c.p.m. (2620/4), a figure equal to that of the 4 active carbon atoms of the pyrrole units (Tables I and III). The activity of any of the 4 methene carbon atoms could be equal to, less than, or greater than the average activity. However, since the radioactivity of the carbon atoms in position 2 of the pyrroles is the same as that of the average of the methene carbon atoms, it appears reasonably certain that these 8 carbon atoms all arise from the α-carbon atom of glycine, and that the radioactivity of the 4 methene carbon atoms and of the carbon atoms in position 2 of the pyrroles is iden-

tical. The location of these carbon atoms in the protoporphyrin molecule is, therefore, in positions $A2$, $B2$, $C2$, and $D2$ and in the 4 carbon atoms of the methene bridges, as shown in Fig. 2. That indeed 8 carbon atoms of the protoporphyrin are derived from the α-carbon atom of glycine was indicated by a previous independent approach (7).[3]

The positions of these carbon atoms in the porphyrin afford a clue to the possible mechanism of condensation of proposed monopyrrole intermediates to form the porphyrin molecule. A pyrrole substituted in the α position by a carbon atom derived from the α-carbon atom of glycine might be such an intermediate. It is known that α-carbon atom-labeled glycine gives rise to serine labeled in the α- and β-carbon atoms (21). Although serine is not utilized in the biosynthesis of protoporphyrin (22),

FIG. 5   FIG. 6

FIGS. 5 AND 6. Hypothetical schemes for porphyrin formation

it is conceivable that a 3-carbon compound, labeled in the α- and β-carbon atoms, may arise from glycine. If this compound were utilized for pyrrole formation with the α-carbon atom of the compound in the α position of the pyrrole and the β-carbon atom of the compound as a side chain on the α position of the pyrrole, a pyrrole would be formed having the structure shown in Fig. 5. If 4 molecules of this pyrrole condensed to form a porphyrin of series III, the mechanism of condensation between rings A and B and B and C would be similar. However, the condensation be-

[3] The number of α-carbon atoms of glycine utilized (N) was calculated from the following formula (see Radin *et al.* (7) for a discussion of the calculation). $N = (N^{15}$ concentration of glycine $\times$ C^{14} activity of porphyrin)/(N^{15} concentration of porphyrin $\times$ C^{14} activity of glycine $\times$ 2) $\times$ 34. By this formula, N was found to be 4 and 8 in Experiments 1 and 2 respectively. We can offer, at present, no explanation for the low value in Experiment 1; low values have occurred very infrequently. However, this discordant value does not affect in any manner the conclusions drawn in the text, since they depend only on the C^{14} activities.

tween rings C and D would involve the elimination of 1 carbon atom, and the condensation between rings D and A would involve another mechanism utilizing a potential "C_1" compound. This would require a total of three different mechanisms for the condensation of the monopyrroles to form a porphyrin. This postulation has one attractive feature however; namely, the ease of formation of porphyrins of the I series. If ring D is rotated 180° so that the α substituent is on the other side, the resulting four pyrroles may now condense by a single mechanism to yield a porphyrin of the I series.

A more uniform mechanism of porphyrin formation is shown in Fig. 6. Four pyrrole intermediates, either unsubstituted in the α positions or bearing substituents which are eliminated, are condensed by a potential C_1 compound. The C_1 compound may be combined to a coenzyme and be utilized in the condensation in a manner analogous to the synthesis of porphyrins described by Andrews, Corwin, and Sharp (23). This method is of interest, since there would exist but one mechanism to condense these similar pyrrole compounds. The potential C_1 compound, the source of the methene carbon atoms, might be related to methanol, formic acid, formaldehyde, or glyoxylic acid, from which CO_2 is lost either prior to or after condensation. Necessarily, this potential C_1 compound must be derived from the α-carbon atom of glycine.

SUMMARY

Whole duck blood was incubated with glycine labeled with C^{14} in the α-carbon atom. The labeled hemin isolated was degraded in order to locate the positions of the carbon atoms in protoporphyrin which are derived from the α-carbon atom of glycine. The hemin was converted by suitable chemical degradation to methylethylmaleimide (derived from the pyrrole rings A and B of the protoporphyrin) and to hematinic acid (derived from the pyrrole rings C and D of the protoporphyrin). The activities of these two compounds were identical. In methylethylmaleimide only carbon atoms $A2$ and $B2$ are active and in hematinic acid only carbon atoms $C2$ and $D2$ are active. These active carbon atoms are in comparable positions in pyrrole rings A and B and in C and D.

The total activity of these carbon atoms ($A2$, $B2$, $C2$, and $D2$) accounts for only 50 per cent of the total activity of the porphyrin. The remaining 50 per cent of the activity must reside in the carbon atoms of the methene bridge. Their average activity is equal to that of the active carbon atoms in the pyrrole rings. It is concluded in the discussion that all 4 carbon atoms of the methene bridge are labeled and that they have equal activities. Therefore 8 carbon atoms in protoporphyrin are derived from

the α-carbon atom of glycine, and these carbon atoms are located in positions $A2$, $B2$, $C2$, and $D2$ and in the 4 carbon atoms of the methene bridges. Hypotheses for porphyrin formation are briefly discussed.

Addendum—In agreement with part of the results of this paper, Muir and Neuberger (24) recently reported in the Proceedings of the Biochemical Society that the α-carbon atom of glycine is utilized for the carbon atoms of the methene bridges of protoporphyrin.

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THE FORMATION OF KETONE BODIES FROM ISOVALERIC ACID*

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In the course of investigations on the biosynthesis of cholesterol, a number of compounds which contained deuterium as a marker were tested as precursors for the steroid structure (1, 2). Aside from acetic acid, which is an efficient precursor (3), two substances, leucine and isovaleric acid, gave rise to high deuterium concentrations in cholesterol (4). Special significance was attached to this finding because these compounds, like cholesterol, contain branched chains. Since leucine and isovaleric acid are ketogenic (5, 6) and also form acetyl groups (4), it was not clear whether the conversion of these compounds to steroids is direct or attributable to the intermediary formation of 2-carbon fragments.

The finding that deuterioisovaleric acid and deuterioleucine gave identical isotope concentrations in cholesterol and N-acetyl groups suggested that isovaleric acid is an intermediate in leucine metabolism (4). Either substance might therefore be used in a study of the fate of these branched chain compounds. It was felt that information on the conversion of these compounds to ketone bodies *in vitro* might be of aid in interpreting their rôle in steroid synthesis. Isovaleric acid appeared to be a more suitable substrate than leucine since it is oxidized more rapidly in liver slices (7).

Isovaleric acid was synthesized to contain C^{13} in the methyl groups and C^{14} in the carboxyl group. The compound was incubated with rat liver slices and the isotope concentrations of the ketone bodies were determined.

EXPERIMENTAL

Preparation of Labeled Compounds

$4,4'-C^{13}$-Isovaleric acid was synthesized by the following series of reactions:

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$$C^{13}H_3I + NaCN \longrightarrow C^{13}H_3CN \longrightarrow C^{13}H_3COOH \longrightarrow (C^{13}H_3)_2CO$$

$$(C^{13}H_3)_2CO + CNCH_2COOC_2H_5 \xrightarrow[H_2]{Pd} (C^{13}H_3)_2CHCHCOOC_2H_5$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad | $$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad CN$$

$$(C^{13}H_3)_2CHCHCOOC_2H_5 \longrightarrow (C^{13}H_3)_2CHCH_2COOH$$
$$\qquad | $$
$$\qquad CN$$

2-C^{13}-Acetic Acid—This was prepared from C^{13}-methyl iodide[1] containing 52.0 atom per cent excess, by way of acetonitrile, as described previously (8).

1,3-C^{13}-Acetone (9)—Acetic acid was converted to its lithium salt and dried thoroughly *in vacuo* at 100°. For pyrolysis, batches of about 2.5 gm. of lithium acetate were placed in Pyrex boats and heated in a Pyrex tube at 350° for 30 minutes and subsequently at 400° for 1½ hours. Before the heating was begun, a slow stream of nitrogen was passed through the reaction tube and this was continued throughout the pyrolysis. Acetone was condensed in a trap which was cooled by dry ice and was used immediately in the next step. In trial runs with normal material, yields of acetone ranged from 80 to 95 per cent. The lithium carbonate formed in the pyrolysis was analyzed for C^{13} and was found to contain 0.09 atom per cent excess C^{13}, or less than 0.2 per cent of the C^{13} concentration of the methyl iodide used.

4,4'-C^{13}-Isovaleric Acid—C^{13}-Acetone was condensed with ethyl cyanoacetate according to the method of Alexander and Cope (10), except that the ethyl cyanoacetate was in excess and it was added in several portions. 0.12 gm. of palladium black, 0.23 gm. of ammonium acetate, and 0.34 ml. of glacial acetic acid were added to a small 3-necked flask equipped with a dropping funnel, an inlet for hydrogen, and a stopper. The C^{13}-acetone was transferred to this flask with the aid of 15 ml. of absolute alcohol. After the flask had been flushed with hydrogen, 1 ml. of ethyl cyanoacetate was added through the dropping funnel and the flask was shaken for hydrogenation. The remainder of the ethyl cyanoacetate was added in 1 ml. portions at 2 hour intervals. Hydrogen uptake ceased after 9½ hours. The catalyst was removed by filtration and 9 gm. of sodium hydroxide in 25 ml. of water were added to the solution containing the ethyl α-cyano-isovalerate. The mixture was heated under a reflux for 26 hours. After removal of alcohol by distillation, the solution was acidified with sulfuric acid and the isopropylmalonic acid was obtained by continuous extraction with ether for 9 hours. The ether extract was dried over sodium sulfate,

[1] Furnished as a contribution by the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

the solvent was distilled, and the remaining liquid was transferred to a small fractionating still. By heating at atmospheric pressure, the malonic acid derivative was decarboxylated and the resulting isovaleric acid distilled. The fraction distilling between 175–180° was collected. In trial runs the yields of isovaleric acid based on acetone ranged from 65 to 76 per cent. The over-all yield of C^{13}-isovaleric acid based on methyl iodide was 52 per cent. The neutral equivalent of an aliquot sample was found to be 103, calculated 102. Since the methyl iodide used as starting material contained 52.0 atom per cent excess C^{13}, the isotope concentration of the synthetic isovaleric acid should be (52.0) (2/5) or 20.8 atom per cent excess. The sodium isovalerate which was analyzed was found to contain 20.9 atom per cent excess.

1-C^{14}-Isovaleric Acid—Isobutyl magnesium bromide was allowed to react with C^{14}-carbon dioxide according to the method of Leslie (11). The reaction was carried out with 3 mM of $C^{14}O_2$, and isovaleric acid was obtained in 89 per cent yield.

For the biological studies, C^{14}-sodium isovalerate and C^{13}-sodium isovalerate were dissolved in water and the mixture brought to dryness. The isotope concentrations of the substrate used in the various experiments are given in Tables I to IV.

Biological Experiments

Incubation—Slices were cut by hand from livers of male rats of the Sprague-Dawley strain weighing about 200 gm. 10 mg. of sodium isovalerate containing C^{13} and C^{14} were dissolved in 20 ml. Krebs' phosphate buffer (12), pH 7.4, and incubated at 37° in the presence of 1.5 gm. (wet weight) of slices for 3 hours. The gas phase was oxygen. In Experiment 2, eight flasks were used. In Experiment 3, the same number of flasks was used but the quantities of tissue and of additions in each flask were doubled. After incubation, the pH of the solution was brought to about 3 with sulfuric acid and the respiratory carbon dioxide was swept into barium hydroxide by a stream of nitrogen.

Isolation of Ketone Bodies, Method I—The liver tissue was removed by centrifugation and the supernatant liquid was treated with copper sulfate and calcium hydroxide. The precipitate was centrifuged and washed with dilute alkali. From the combined supernatant and washings acetone was isolated as the mercury complex, according to Van Slyke (13). This fraction includes any acetone that might be formed biologically and acetone formed by chemical decarboxylation of acetoacetate, and will be called "combined" acetone. While the solution was heated with mercuric sulfate, the CO_2 resulting from the decarboxylation of acetoacetate was swept from the reaction flask into barium hydroxide by a stream of nitrogen.

Method II—The isotope concentrations of the individual carbon atoms of acetoacetate were determined by degrading acetoacetate according to the method of Weinhouse and Millington (14). On treatment with acid permanganate in the cold, acetoacetate is oxidized to CO_2, formic acid, and acetic acid which arise from carbon atoms 1, 2, and 3 and 4 respectively. Acetone and isovaleric acid are not oxidized under these conditions. This method permits separation of acetone, which might be formed biologically, from acetone resulting from the chemical decarboxylation of acetoacetate.

The filtrates obtained after deproteinization of the incubation mixtures were oxidized by acid permanganate at 0°, and the volatile material was removed by steam distillation. The distillate was neutralized and redistilled in order to separate any neutral compounds such as acetone from the volatile acids. The acetone isolated after permanganate oxidation will be called "free" acetone. In Experiment 2, a known quantity of normal acetone was added as carrier to the neutral distillate and acetone was precipitated. In Experiment 3, the acetone in the steam distillate was determined colorimetrically as the 2,4-dinitrophenylhydrazone (15). An equal quantity of non-isotopic acetone (5 mg.) was added, the solution was neutralized and distilled, and acetone in the distillate was precipitated with the mercury-sulfate reagent.

The fraction containing the volatile acids was heated with mercuric sulfate in acid solution, and the CO_2 resulting from the oxidation of formic acid was collected as barium carbonate. The remaining solution containing acetic acid and any unchanged substrate was extracted continuously with petroleum ether. To insure complete removal of the isotopic isovaleric acid, a large excess of normal isovaleric acid was added and the extraction with petroleum ether was repeated. The aqueous solution was then steam-distilled, and the acetic acid in the distillate was isolated as the silver salt. This was recrystallized several times.

The mercury-acetone precipitates were purified by dissolving in 10 ml. of N hydrochloric acid, distilling of the acetone, and reprecipitating with mercury sulfate. In Experiment 3 an aliquot of the acetone in the distillate was isolated as the 2,4-dinitrophenylhydrazone.
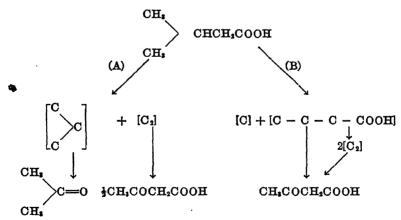
Total ketone bodies were determined by mercury precipitation of an aliquot of the incubation mixture in Experiment 3 in which 1.28 mM of sodium isovalerate had been used. The quantity of mercury-acetone complex corresponded to 0.56 mM of acetone. The unchanged substrate was recovered by petroleum ether extraction of the acidified reaction mixture after steam distillation. By titration of the acid in the extract 0.13 mM of acid was found. Therefore 1.15 mM, or 90 per cent of the substrate, were metabolized. The quantity of ketone bodies formed from isovaleric acid can be roughly estimated from the average relative isotope concentra-

tion of all carbon atoms of acetoacetate. This is given by the relative isotope concentrations of the "combined" acetone and the carboxyl carbon of acetoacetate (Table III): $(70 + 70 + 11 + 14)/4$ or 41. Thus 41 per cent of the 0.56 mM of ketone bodies, or 0.23 mM, was derived from 1.15 mM of substrate. This value represents the minimal conversion of isovaleric acid to ketone bodies, since β-hydroxybutyric acid, which was not determined, must have been present.

Isotope Analyses—The samples of mercury acetone, 2,4-dinitrophenyl-hydrazone, and iodoform were converted to CO_2 by wet combustion according to Van Slyke and Folch (16). All other samples were burned in a micro combustion apparatus. For C^{14} determinations, barium carbonate samples were transferred to aluminum cups of 3.5 sq.cm. area with the aid of methyl alcohol. The samples were counted with a flow gas counter for a sufficient length of time to insure a probable error of less than 5 per cent. Counts were corrected for thickness, according to the method of Reid (17), and results are given as counts per minute of infinitely thick samples of barium carbonate. For C^{13} determinations the barium carbonate samples were converted to CO_2 in a vacuum system, according to Rittenberg (18), and the isotope concentrations were determined in a mass spectrometer.

RESULTS AND DISCUSSION

Isovaleric acid is known to be a ketogenic compound and a source of acetyl groups. Two pathways have been suggested to account for the formation of these products (4).

In the present experiments, ketone body formation from isovaleric acid containing C^{13} in the methyl groups and C^{14} in the carboxyl group was studied. It was felt that the isotope distribution in the ketone bodies re-

sulting from the doubly labeled substrate might aid in the elucidation of the reaction mechanism.

In Experiment 1, the total ketone bodies obtained after incubation of rat liver slices with isovalerate were isolated as the mercury-acetone complex. In Experiments 2 and 3, the ketone bodies were divided into two portions. One portion was treated with mercury-sulfate reagent to yield the total ketone bodies in the form of the mercury-acetone complex ("combined" acetone). The other portion was oxidized with cold acid permanganate in order to separate from acetoacetate any acetone ("free" acetone) which might have been formed directly, and in order to determine the isotope concentrations in each carbon atom of acetoacetate.

The results obtained are shown in Tables I, II, and III. In Column 1 are given the isotope concentrations which were actually found. In Column 2 are given the isotope concentrations of the labeled atoms only of the substances isolated. For example, the isotope concentrations for acetone in Experiment 1, Table I, were calculated in the following manner. The C^{13} concentration in the methyl carbons of acetone was obtained by multiplying the found value by $3/2$, since only the methyl carbons contain C^{13}. The value thus obtained is (26.8) $(3/2)$ or 40.2. The C^{14} concentration in the carbonyl carbon of acetone was obtained by multiplying[2] the found value by 3. The relative isotope concentrations (RIC) in Column 3 were obtained by dividing the values of Column 2 by the isotope concentration of the labeled atoms in the substrate molecule and multiplying by 100. Thus the RIC for the methyl carbons of acetone, Experiment 1, is $(40.2/47.2)$ (100) or 85. These RIC values therefore represent the isotope concentrations in the labeled carbon atoms of the reaction products based on 100 atom per cent excess C^{13} or a specific activity of 100 in the labeled atoms of the substrate.

The relative isotope concentrations of C^{13} of the "combined" acetone, *i.e.* acetone obtained by treatment of the incubation mixture with mercuric sulfate reagent (Method I), were 85, 51, and 70 in the three experiments (Tables I to III). The maximal isotope concentrations of C^{13} in the methyl carbons of any acetone formed according to Pathway A would be 100. If, on the other hand, isovalerate had been "demethylated" and acetoacetate had been formed from the 4-carbon intermediate, one of the methyl carbons of isovaleric acid would have been lost. Acetone formed by this route could not contain more than 1 atom of C^{13} per molecule; *i.e.*, a relative isotope concentration of 50, if it is assumed that acetoacetate formed by Pathway B would be equally derived from carbon atoms 1 and 2, and

[2] In one experiment the acetone was degraded to iodoform. This iodoform contained no C^{14} and contained C^{13} in concentrations which accounted for the C^{13} content of the total acetone molecule.

TABLE I

Formation of Ketone Bodies in Rat Liver Slices from C^{13}, C^{14}-Isovalerate

47.2 atom per cent excess C^{13} in methyl carbons; specific activity 1.1×10^6 c.p.m. of C^{14} in carboxyl carbon.

Experiment 1	Isotope concentration		
	Atom per cent excess or counts per min.		Relative isotope concentrations
	Found (1)	Calculated for labeled carbon atoms (2)	(3)
"Combined" acetone, C^{13}	26.8	40.2*	85
" " C^{14}	27,000	81,000†	7.4

* Methyl carbons of acetone.

† Carbonyl carbon of acetone.

TABLE II

Formation of Ketone Bodies in Rat Liver Slices from C^{13}, C^{14}-Isovalerate

7.5 atom per cent excess C^{13} in methyl carbons, specific activity 1.4×10^5 c.p.m. in C^{14} in carboxyl carbon.

Experiment 2	Isotope concentration					
	C^{13}			C^{14}		
	Atom per cent excess		Relative isotope concentrations	Counts per min.		Relative isotope concentrations
	Found (1)	Calculated for labeled carbon atoms (2)	(3)	Found (1)	Calculated for labeled carbon atoms (2)	(3)
"Combined" acetone	2.56	3.84*	51	3,700	11,100†	8.0
"Free" acetone‡	0.88	1.32*	18	1,000	3,000†	2.1
Acetic acid (C-3 and C-4 of acetoacetate)	0.87	1.74*	23	3,900	7,800§	5.6
Formic acid (C-2 of acetoacetate)	1.45	1.45	19			
Acetoacetate carboxyl				15,000	15,000	11
Respiratory CO_2	0.03	0.03	0.4	6,800	6,800	4.9

* Methyl carbons of acetone or acetic acid.

† Carbonyl carbon of acetone.

‡ Diluted with carrier acetone and cannot be compared directly to other compounds in the table.

§ Carboxyl carbon of acetic acid.

3 and 4 of isovaleric acid. The high C^{13} concentrations found in acetone therefore show that it contained more than 1 atom of C^{13} per molecule, and thus indicate that the ketone bodies were formed in accordance with Pathway A.

In Experiments 2 and 3, three separate fractions of ketone bodies were obtained, one representing total ketone bodies ("combined" acetone, Method I), the second acetoacetate, and the third "free" acetone (Method

TABLE III

Formation of Ketone Bodies in Rat Liver Slices from C^{13},C^{14}-Isovalerate

6.2 atom per cent excess C^{13} in methyl carbons, specific activity 1.05×10^5 c.p.m. of C^{14} in carboxyl carbon.

Experiment 3	Isotope concentration					
	C^{13}			C^{14}		
	Atom per cent excess		Relative isotope concentrations	Counts per min.		Relative isotope concentrations
	Found (1)	Calculated for labeled carbon atoms (2)	(3)	Found (1)	Calculated for labeled carbon atoms (2)	(3)
"Combined" acetone.....	2.88	4.32	70	3,800	11,400	10.9
"Free" acetone,* Hg complex..................	0.95	1.43	23	1,000	3,000	2.9
"Free" acetone,* 2,4-dinitrophenyl hydrazone*†	0.91	1.36	22	900	2,700	2.6
Acetic acid (C-3 and C-4 of acetoacetate).......	1.55	3.10	50	4,400	8,800	8.4
Formic acid (C-2 of acetoacetate)...............	3.55	3.55	57	360	360	0.3
Acetoacetate carboxyl....	0.05	0.05	0.8	15,000	15,000	14
Respiratory CO_2.........	0.04	0.04	0.6	12,700	12,700	12

* Diluted with carrier acetone.
† Calculated for acetone.

II). In both experiments, the acetic acid derived from carbon atoms 3 and 4, and formic acid derived from carbon atom 2 of acetoacetate, contained C^{13}, although in lower concentrations than the "combined" acetone. The presence of C^{13} in acetoacetate appears to be inconsistent with Pathway A because, according to this scheme, the carboxyl and α-carbon atoms of isovalerate should be the only sources of acetoacetate. The question arises as to whether acetone is indeed a primary product of the oxidation of isovalerate or is formed secondarily by decarboxylation of acetoacetate. In any acetone resulting from the decarboxylation of acetoacetate, the methyl carbons could not have an isotope concentration greater than the

average of the methyl and methylene carbons of acetoacetate. In Experiment 2, the latter values are $(23 + 19)/2$, or 21, as compared to a value of 51 in the methyl carbons of acetone. In Experiment 3, the corresponding values are $(50 + 57)/2$ or 54 as compared to 70 in the methyl carbons of acetone. These data suggest that the acetone isolated by Method I is not formed exclusively by decarboxylation of acetoacetate.

C^{14} was present in all acetone fractions as well as in the carboxyl and the carbonyl carbon atoms of acetoacetate, indicating that some acetoacetate was formed by condensation of a fragment derived from the carboxyl moiety of isovalerate. The RIC values for C^{14} in the acetone fractions and in the carboxyl carbon of acetoacetate were much lower than the RIC values for C^{13} in acetone. The data indicate that, of the total ketone bodies formed from isovalerate, only one-third to one-sixth was derived from the

<div align="center">

TABLE IV

Relative Isotope Concentrations of C^{13} and C^{14} in Ketone Bodies

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	Relative isotope concentrations $C^{13}:C$	
	Experiment 2	Experiment 3
Acetoacetate $\frac{1}{2}(CH_3 + CH_2):CO$	3.8	6.4
"Combined" acetone $CH_3:CO$	6.4	6.4
"Free" acetone, Hg complex $CH_3:CO$	8.6	7.9
" " 2,4-dinitrophenylhydrazone $CH_3:CO$		8.5

carboxyl fragment. Irrespective of the mechanism involved, it is clear that under the present experimental conditions the isopropyl moiety of isovalerate is more readily converted to ketone bodies than the carboxyl fragment. It should also be pointed out that the respiratory carbon dioxide contained 10 to 20 times as much C^{14} as C^{13}. This may indicate that the carboxyl fragment of the molecule enters much more rapidly into oxidative reactions than the isopropyl moiety.

A further indication that some acetone is formed directly from isovalerate is given by the $C^{13}:C^{14}$ ratios in the ketone bodies. If the acetone were derived exclusively from acetoacetate, the $C^{13}:C^{14}$ ratios in the "combined" acetone should be the same as those in the corresponding carbon atoms of acetoacetate; that is, $CH_3:CO$ of acetone = $\frac{1}{2}(CH_3 + CH_2):CO$ of acetoacetate. This ratio was 50 per cent greater in the "combined" acetone in Experiment 2 but was the same in Experiment 3 (Table IV).

Additional attempts to establish a direct formation of acetone from isovalerate were made in the following manner. Acetone remaining after oxidation of the ketone bodies with permanganate was isolated and its

isotope concentration was determined. This acetone fraction ("free" acetone) would contain any acetone formed directly from the isopropyl group of isovalerate and also any acetone formed by spontaneous decarboxylation of acetoacetate prior to oxidation by permanganate. The quantity of acetone obtained by this procedure was too small for analysis, and it was necessary to dilute with a large quantity of normal acetone. In Experiment 2, the amount of the "free" acetone was not determined and therefore the original isotope concentration of this fraction could not be calculated. In Experiment 3, "free" acetone was determined colorimetrically and then diluted with normal acetone before isotopic analysis. The isotope concentrations for the "free" acetone, which were calculated from the colorimetric analysis and from the amount of carrier added, gave values only one-third of that of the "combined" acetone in the same experiment. Therefore, it appears likely that this fraction contained neutral ketones other than acetone. For this reason, the absolute isotope concentrations in the "free" acetone cannot be compared to those of the "combined" acetone or of the acetoacetate fractions.[3] However, the $C^{13}:C^{14}$ ratios should indicate the relation of these acetone fractions to acetoacetate. These ratios are significantly higher for the "free" acetone than for either the "combined" acetone or for carbon atoms 2, 3, and 4 of acetoacetate (Table IV). These differences suggest strongly that a 3-carbon compound is formed directly from the isopropyl group of isovaleric acid. The formation of such an intermediate is not consistent with Pathway B.

The fact that the quantity of "free" acetone was small compared to the total quantity of ketone bodies, and that the C^{13} concentration in acetoacetate was relatively high, can best be explained by assuming that this 3-carbon intermediate is rapidly metabolized further. Evidence indicating that acetone can be oxidized to an intermediate which has the properties of acetate has been reported recently. Borek and Rittenberg (19) have found that labeled acetone can serve as a precursor of cholesterol in isolated liver, and have suggested that the effect may be due to the conversion of acetone to acetate. According to Price and Rittenberg (20), acetone is converted in intact animals to acetyl groups which can acetylate foreign amines. Thus the appearance of C^{13} in the methyl and methylene carbons of acetoacetate on oxidation of isovalerate *in vitro* could reasonably result from the conversion of acetone to a 2-carbon intermediate which is capable of condensing to form acetoacetate. It is not probable that a carboxylation of acetone is responsible for the appearance of C^{13} in aceto-

[3] In one experiment "free" acetone was isolated without dilution by carrier. The RIC value for C^{13} was 48; that is, of similar magnitude to that in the "combined" acetone in Experiments 2 and 3.

acetate, since Swenseid *et al.* (21) failed to detect labeled ketone bodies following the administration of isotopic carbon dioxide to fasted rats.

It is clear from the results of the present investigation that the formation of ketone bodies from isovaleric acid cannot be explained entirely on the basis of either of the two schemes outlined above. The two suggested metabolic pathways for isovalerate differ in that acetone as well as acetoacetate is formed as reaction products according to Pathway A, while, according to Pathway B, acetoacetate alone is formed. If the assumption were correct that acetone is a primary oxidation product, its detection might be difficult because of further conversion to acetoacetate. In this case the mechanism would be obscured because both pathways would lead to the same end-products. Nevertheless, the experimental findings are in better accord with a reaction mechanism in which isovaleric acid is oxidized initially at the β position to yield a 3-carbon and a 2-carbon particle. This conclusion is based on the findings that the C^{13} concentrations in the methyl carbons of acetone were high on an absolute basis and also high compared to that in the methyl or methylene carbons of acetoacetate. Secondly, the $C^{13}:C^{14}$ ratios were significantly greater in the acetone fractions than in the corresponding carbon atoms of acetoacetate. This finding can be explained only if acetone is formed directly from isovaleric acid, but it is at variance with the assumption that acetone arose exclusively by decarboxylation of acetoacetate. Although the evidence presented suggests that isovaleric acid is initially oxidized at the β-carbon atom to yield a 3- and a 2-carbon fragment, the simultaneous occurrence of the "demethylation" mechanism cannot be excluded.

It is possible that the postulated 3-carbon intermediate is not acetone, but that acetone is formed in a side reaction from a more labile 3-carbon precursor. In the following paper evidence is presented to show that *in vivo* acetone is indistinguishable from acetate as a precursor for cholesterol. On the other hand, the isopropyl group of isovaleric acid is a more efficient source of carbon atoms for cholesterol synthesis than acetone or acetate, and it appears therefore that the primary intermediate in the oxidation of isovalerate is a 3-carbon compound other than acetone (22).

Oxidative deamination and decarboxylation to the fatty acid containing 1 less carbon atom are generally believed to be steps in the degradation of amino acids (23). It might be expected that the formation of ketone bodies from leucine and isovaleric acid follows identical pathways. The conversion of leucine to acetoacetate has recently been studied by Coon and Gurin (24) under similar conditions. The results of these authors agree with those presented here in some respects but not in others. Thus, 1-C^{14}-isovalerate as well as 3-C^{14}-leucine yielded acetoacetate in which both the

acetyl and the acetate moiety contained similar concentrations of C^{14}. This indicates that carbon atoms 1 and 2 of isovalerate and carbon atoms 2 and 3 of leucine can be the sources of a 2-carbon fragment of similar properties. On the other hand, the results pertaining to the metabolism of the isopropyl portion of leucine and of isovaleric acid are not in agreement. Coon and Gurin found that the acetoacetate which was formed from 4-C^{14}-leucine was not symmetrically labeled. Isotope was present only in the carbonyl atom. In contrast, in the present experiments the isovaleric acid which contained C^{13} in the methyl groups gave rise to acetoacetate with similar C^{13} concentrations in the methyl and methylene carbon atoms. While the results obtained with leucine suggest that the isopropyl portion of leucine is a precursor of the acetyl moiety of acetoacetate only, it is evident from our findings that the isopropyl group of isovaleric acid is a source of all carbon atoms of acetoacetate.

SUMMARY

1. The preparation of 4,4'-C^{13}-isovaleric acid has been described.

2. Rat liver slices were incubated in the presence of a mixture of 4,4'-C^{13}-isovaleric acid and 1-C^{14}-isovaleric acid. The resulting ketone bodies were isolated and their isotope concentrations determined.

3. Evidence has been obtained to show that isovaleric acid is oxidized initially at the β-carbon atom to yield a 3-carbon and a 2-carbon intermediate and that both intermediates are metabolized further to yield acetoacetate.

4. The isopropyl portion of isovalerate is more rapidly converted to ketone bodies and less rapidly oxidized to CO_2 than the carboxyl moiety.

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THE UTILIZATION OF ISOVALERIC ACID FOR THE SYNTHESIS OF CHOLESTEROL*

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The presence of branched chains in the amino acids leucine and valine and in the steroids has led to the speculation that a biochemical relationship might exist between these substances. Previous work had demonstrated that the feeding to rats of deuterioleucine and deuterioisovaleric acid but not of deuteriovaline or of deuterioisobutyric acid results in the formation of labeled cholesterol (1). Since leucine and isovaleric acid form 2-carbon compounds in intermediary metabolism, and since acetate is a precursor of cholesterol (2), it was not clear whether leucine and isovaleric acid play a specific rôle in sterol synthesis or are merely sources of acetate. The utilization of a branched chain compound in steroid synthesis has now been reinvestigated with the aid of isovaleric acid which contained C^{13} in the methyl groups and C^{14} in the carboxyl position. Some indications as to the metabolism of this compound have been obtained by studying its conversion to ketone bodies *in vitro* (3). The results suggested that a 3- and a 2-carbon fragment were the primary oxidation products of isovaleric acid. The object of the experiments which are reported here was to investigate whether the utilization of isovaleric acid for cholesterol synthesis could be attributed to the intermediary formation of these products.

In the feeding experiments with labeled isovaleric acid, both the synthesis of cholesterol and the formation of acetate from the isotopic substrate were studied. By feeding simultaneously foreign amines which are excreted as N-acetyl derivatives, the conversion of isovaleric acid to 2-carbon compounds could be measured. Since the possibility existed that acetone was an intermediate in the breakdown of isovalerate, it became neces-

sary to examine acetone as a precursor of acetyl groups and of cholesterol. Moreover, acetate labeled in both the carboxyl and methyl carbon atoms was fed for purposes of comparison.

The rats received a stock diet containing the labeled compound, and either phenyl-L-aminobutyric acid or p-aminobenzoic acid, for various periods of time. The N-acetylamino acids were isolated from the urine, and cholesterol and fatty acids were obtained from the liver.

EXPERIMENTAL

Synthesis of 4,4'-C^{13}- and 1-C^{14}-Isovaleric Acids—The preparation of these compounds is described in the preceding paper (3).

Synthesis of 1,3-C^{14}-Acetone and 1-C^{13}-Acetic Acid—1,3-C^{14}-Acetone was prepared from 2-C^{14}-acetate as described (3). 1-C^{13}-Acetic acid was synthesized by way of acetonitrile according to Weinhouse *et al.* (4). 1-C^{13},-2-C^{14}-Acetate was obtained by mixing the two singly labeled compounds.

Feeding Experiments—Rats of the Sprague-Dawley strain weighing about 200 gm. were fed 12 gm. per day of the following diet: 68 per cent cornstarch, 19 per cent casein, 5 per cent dried yeast, 5 per cent salt mixture,[1] 1 per cent cod liver oil, and 2 per cent Mazola oil. The labeled sodium isovalerate, or sodium acetate, was dissolved in water and either 100 mg. of phenyl-L-aminobutyric acid or 100 mg. of p-aminobenzoic acid per 100 gm. of body weight were mixed with the diet. In Experiments 2, 3, and 4 the cod liver oil and Mazola oil were omitted, and 1 drop of methyl linoleate was added per day. The labeled acetone and acetate in Experiment 3 were dissolved in 3 ml. of water, and 0.5 ml. portions were administered by stomach tube in the morning, afternoon, and evening for 2 days.

Isolation of Urinary and Body Constituents—Acetyl-L-phenylaminobutyric acid and acetyl-p-aminobenzoic acid were isolated from the pooled urine which was excreted during the experimental period, and the samples were purified as described by Bloch and Rittenberg (5). The animals were killed by exsanguination after stunning, and liver cholesterol was isolated by standard procedures. The saturated fatty acids were separated by way of the lead soaps (6).

Isotope Analyses—All isolated compounds were converted to CO_2 in a micro combustion apparatus at 900°. The isotope analyses were carried out as previously described (3). The C^{14} values are expressed as counts per minute of infinitely thick samples of $BaCO_3$.

RESULTS AND DISCUSSION

The study of acetate metabolism and of acetate formation in animal tissues has been greatly aided by the observation of Bernhard that acetic

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 15, 311 (1913).

acid is a source of acetyl groups in the acetylation of foreign amines (7). Frequent use has been made of this finding in order to study the formation of acetic acid from a labeled substrate (8–10). For quantitative measurements the formation of acetyl groups from a labeled test substance may be compared to acetyl formation from labeled acetic acid itself. In previous experiments in which both labeled acetate and a foreign amine were fed, the isotope concentration in the excreted acetyl groups was proportional to the amount of acetate administered (5). If a labeled precursor gives rise to N-acetyl groups of an isotope concentration similar to that yielded by an equivalent quantity of labeled acetate, it may be concluded that

TABLE I

Isotope Concentrations in N-Acetyl, Cholesterol, and Saturated Fatty Acids of Rats Fed 4,4'-C^{13}, 1-C^{14}-Isovalerate Containing 18.9 Atom per Cent Excess C^{13}, Specific Activity 1.7 × 10^5 C.p.m. of C^{14} in Total Molecule

Experiment No.	Days	Quantity of isovalerate fed, mм per 100 gm. per day	Isotope	Isotope concentration					
				Acetyl group of excreted acetylamine		Liver cholesterol		Saturated fatty acids of liver	
				Found* (1)	RIC (2)	Found* (1)	RIC (2)	Found* (1)	RIC (2)
1†	2	0.5	C^{13}	0.63	2.68	0.43	1.83	0.15	0.64
			C^{14}	2400	0.56	800	0.19	700	0.16
2	8	0.125	C^{13}	0.19	0.81	0.19	0.81	0.11	0.47
			C^{14}	1250	0.29	600	0.14	100	0.24

* Atom per cent excess C^{13} or counts per minute C^{14}.

† The foreign amine fed was p-aminobenzoic acid. In all other experiments Phenyl-L-aminobutyric acid was fed.

acetate is the main product. Since the isovaleric acid used here was labeled by two different carbon isotopes, the formation of acetyl groups from different portions of the molecule could be compared in the same experiment.

The results of two feeding experiments with isovalerate are shown in Table I. The isotope concentrations given in Column 1 are those actually found, expressed as atom per cent excess for C^{13} and counts per minute for C^{14}. The values given in Column 2 (RIC, relative isotope concentration) were obtained by dividing the observed isotope concentrations of the various isolated compounds by the isotope concentration calculated for a 2-carbon particle arising from either carbon atoms 1 and 2 or 3 and 4 of isovalerate. Thus, since the C^{13} concentration at carbon atoms 4 and 4' of isovalerate was 47.0 atom per cent excess, and since carbon atom 3 contained no excess of isotope, a 2-carbon fragment derived from carbon atoms 3 and 4 would have an average isotope concentration of 23.5 atom per cent

excess C^{13}.   Similarly, since the C^{14} concentration of the carboxyl carbon was 850,000 c.p.m., and since carbon atom 2 was not labeled, the 2-carbon particle consisting of carbon atoms 1 and 2 of isovalerate would have an average specific activity of 425,000 c.p.m.   In Experiment 3, Table II, the RIC values for C^{14} were based on a 2-carbon particle derived from acetone and containing 3000 c.p.m.   The experimental data were recalculated in this manner in order to allow a direct comparison with results obtained after the feeding of labeled acetate.

When doubly labeled isovaleric acid was fed for 2 days at a level of 0.5 mM per 100 gm. of rat weight (Experiment 1, Table I), the RIC for C^{13} in the excreted N-acetyl groups was found to be identical with that obtained after feeding equivalent amounts of acetate (Table II).   When the feed-

TABLE II

Isotope Concentration in N-Acetyl, Cholesterol, and Saturated Fatty Acids of Rat Fed
1,3-C^{14}-Acetone, Specific Activity 4500 C.p.m. of C^{14}, and 1-C^{13}-Acetate, 9.8 Atom
per Cent Excess C^{13}

Experiment 3, 2 days; fed 0.5 mM per 100 gm. per day of each compound.

	Isotope concentration					
Compounds fed	Acetyl group of excreted acetylamine		Liver cholesterol		Saturated fatty acids of liver	
	Found* (1)	RIC (2)	Found* (1)	RIC (2)	Found* (1)	RIC (2)
1,3-C^{14}-Acetone	700	2.33	70	0.23	175	0.58
1-C^{13}-Acetate	0.26	2.65	0.026	0.26	0.086	0.88

* Atom per cent excess C^{13} or counts per minute of C^{14}.

ing period was 8 days and the dosage of isovalerate was reduced to one-fourth of that in Experiment 1, the RIC for C^{13} in the acetyl groups was 0.81, or slightly more than one-fourth of that in Experiment 1.   Therefore the isopropyl moiety of isovaleric acid is converted to acetyl groups to very nearly the same extent as an equivalent quantity of acetate.

That the degradation product of the isopropyl group of isovalerate has properties similar to those of acetate becomes evident also on comparison of the isotope concentrations in the fatty acids which were isolated after the feeding of isovalerate and of acetate.   The RIC values for the saturated fatty acids from the two experiments do not differ by more than 30 per cent (see Experiments 1 and 3).   On the other hand, the effects of acetate and isovalerate in cholesterol synthesis differ markedly.   The isopropyl group of isovaleric acid gave rise to cholesterol which contained 7 times as much isotope as the sterol resulting from the feeding of an equivalent amount of acetate (Experiment 1, Table I; Experiment 3, Table II).

In previous experiments, when labeled acetate was fed for 1 week or less, it had been observed that the isotope concentration of the saturated fatty acids of liver is appreciably higher than that of cholesterol in the same organ (10, 11). Only in experiments extending over a period of a month will the isotope concentration of cholesterol approach that of the fatty acids (12). These relationships are explainable on the basis of a greater rate of turnover of liver fatty acids as compared to the turnover of cholesterol. Thus in Experiment 3, Table II, after acetate feeding the RIC of the fatty acids is more than 3 times as high as that in cholesterol.

In contrast, this relationship is reversed after the feeding of isovalerate. The isotope concentration of cholesterol in Experiment 1, Table I, is 3 times as high as that in the fatty acids, and almost twice as high as in the fatty acids in Experiment 2. It is clear from these findings that the isopropyl portion of isovaleric acid behaves in a manner which is indistinguishable from that of acetate with respect to the acetylation reaction and to fatty acid synthesis, but is a much more efficient precursor for cholesterol than acetate.

It should be pointed out that in the present experiments the reactions of methyl-labeled isovalerate have been compared with those of carboxyl-labeled acetate. Experiments *in vitro* with rat liver and doubly labeled acetate have shown that the methyl and carboxyl carbons are not equally utilized for cholesterol synthesis, but in a ratio of 1.27 (13). In order to ascertain whether the same relationship holds *in vivo*, doubly labeled acetate was fed and cholesterol was isolated from the liver. As in the experiments *in vitro*, the methyl carbons of acetate were utilized to a somewhat greater extent than the carboxyl carbons (Table III).[2] The efficiency of the isopropyl group of isovalerate must therefore be compared to that of the methyl group of acetate. On this basis it is found that the isopropyl group of isovalerate affords an isotope concentration in cholesterol which is more than 5 times greater than that expected from acetate.

The utilization of the carboxyl moiety of isovalerate for the various synthetic reactions can be similarly evaluated by comparison with acetate and with the isopropyl fragment of isovalerate. The data in Experiments 1 and 2 (Table I) show that the RIC values for C^{14} in the N-acetyl groups are only one-fifth and one-third of those given by the isopropyl fragment of isovalerate or by acetate. Hence, the carboxyl group of isovalerate is a much poorer source of acetyl groups. The utilization of the carboxyl

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[2] The two carbon atoms of acetate are utilized to the same extent in the formation of acetyl groups and of the fatty acids. Glycogen from the liver and hemin from the blood were also isolated. The methyl and carboxyl carbons of acetate were utilized in a ratio of 1.8 in the synthesis of glycogen, and in a ratio of 3.1 in the synthesis of hemin.

moiety of isovaleric acid for the synthesis of the higher fatty acids and of cholesterol is also low. The values obtained were much smaller than those resulting from acetate or from the isopropyl group of isovaleric acid.

In rat liver slices, the isopropyl moiety of isovalerate formed ketone bodies at a much faster rate than the carboxyl fragment. On the other hand, under the same conditions the carboxyl fragment was much more rapidly oxidized to CO_2 (3). These differences in the metabolism of the two portions of the isovaleric acid molecule are also reflected in the present experiments *in vivo*. In the synthetic reactions which have been studied here, the isopropyl portion of isovalerate is a much more efficient carbon source than the carboxyl portion of the molecule. It appears, therefore, that the oxidation of this fatty acid yields 2-carbon fragments which enter into various metabolic reactions at different rates.

TABLE III

Isotope Concentration in N-Acetyl, Cholesterol, and Saturated Fatty Acids of Rats Fed
1-C^{13},2-C^{14}-Acetate, 25.99 Atom per Cent Excess C^{13},
Specific Activity 65,000 C.p.m.

Experiment 4, 7 days; fed 1 mM per 100 gm. per day.

Isotope	Isotope concentration					
	Acetyl group of excreted acetylamine		Liver cholesterol		Saturated fatty acids of liver	
	Found* (1)	RIC (2)	Found* (1)	RIC (2)	Found* (1)	RIC (2)
C^{14}	5400	8.31	1900	2.93	2200	3.38
C^{13}	2.22	8.54	0.59	2.27	0.81	3.12

* Atom per cent excess C^{13} or counts per minute of C^{14}.

The experiments on ketone body formation described in the preceding paper raised the possibility that acetone was an intermediate in the metabolism of isovalerate. In order to determine whether acetone was responsible for the effect shown by the isopropyl portion of isovalerate in cholesterol synthesis, 1,3-C^{14}-acetone and 1-C^{13}-acetate were administered simultaneously. The two test substances did not differ significantly in their ability to form labeled acetyl groups, fatty acids, or cholesterol (Table II). It appears therefore that acetone is rapidly oxidized to the same intermediate which arises from acetate. These data confirm those of Borek and Rittenberg (14) on the utilization of acetone for cholesterol synthesis *in vitro*. Thus, the effect of isovalerate in cholesterol synthesis cannot be ascribed to the formation of acetone as an intermediate.

It is evident from the data presented that the isopropyl portion of isovalerate gives rise to an intermediate which behaves like acetate in the

acetylation reaction and in fatty acid synthesis, but is an exceptionally efficient source of carbon atoms for cholesterol. This conclusion is based on the finding that the incorporation of isopropyl carbon into cholesterol is high not only on an absolute basis but also relative to the C^{13} concentration in the fatty acids and the acetyl groups. If a 2-carbon fragment derived from the isopropyl moiety were responsible for the effect shown by isovalerate in cholesterol synthesis, the properties of this 2-carbon intermediate would have to differ from those of the 2-carbon intermediate formed from acetate, pyruvate (10), acetone, or the 2-carbon fragment arising from carbon atoms 1 and 2 of isovalerate.

One of the objects of the present investigation was to test whether the branched structures in the cholesterol molecule have their origin in similar groups which are available to the animal organism. The results with isovaleric acid are consistent with the view that one of the intermediates involved is a 3-carbon compound derived from the isopropyl portion of the molecule. In addition, some of the isopropyl carbons must enter into cholesterol by way of a 2-carbon fragment similar to acetate. Acetic acid has been shown to serve as the carbon source for the isopropyl group of the aliphatic side chain and for the angular methyl groups of cholesterol. It was suggested that 2 molecules of acetate yield a 3-carbon unit to form these groupings (13). In view of the results obtained with acetate, the isopropyl portion of isovaleric acid cannot be an exclusive precursor for these same carbon atoms of the steroid molecule but it may perhaps provide an independent carbon source. Whether a 3-carbon compound derived from isovaleric acid is specifically used for other portions of the cholesterol molecule cannot be decided on the basis of the present data.

SUMMARY

1. Doubly labeled isovalerate and foreign amines were fed to rats. The acetylamine was isolated from the urine, and cholesterol and saturated fatty acids were obtained from the liver.

2. The isopropyl portion of isovalerate forms acetyl groups and fatty acids to the same extent as does acetate but is a much more efficient source of carbon atoms for cholesterol.

3. The carboxyl portion of isovaleric acid is less efficiently converted to acetyl groups, fatty acids, or cholesterol than either the isopropyl portion of isovalerate or acetate.

4. After the administration of labeled acetone, the excreted acetyl groups, fatty acids, and cholesterol showed the same isotope concentrations as were observed after the feeding of labeled acetate. This result indicates that acetone is rapidly converted to a 2-carbon compound.

5. C^{13}, C^{14}-Acetate was fed in order to compare the utilization of the 2 carbon atoms of acetate in these synthetic reactions.

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AN IMPROVED METHOD FOR THE PREPARATION OF PROTECTIVE PSEUDOGLOBULIN*

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Experiments indicating the existence of a menstrual toxin responsible for some of the clinical features of preeclamptic and eclamptic toxemias of pregnancy, and of a protective factor capable of neutralizing this toxin in animal experiments and in human cases have been reported by Smith and Smith (1-3). The apparent success obtained in some of the clinical cases in which protective pseudoglobulin (PPs) was administered indicated the desirability of a method for the preparation of the material in higher yield and purity. The work of Cohn and his colleagues on the fractionation of human plasma in ethanol-water mixtures at low temperature, advantage being taken of pH and ionic strength, offered a means whereby this end might be accomplished.

Materials and Methods

Exudate was collected from paracenteses and thoracenteses in human cases of carcinomatosis into clean, pyrogen-free bottles. It was freed of clot and cells by sedimentation, and stored frozen at $-5°$ until used. Processing was carried out at the Harvard pilot plant by means of the techniques mentioned above. These methods have been fully described by Cohn *et al.* (4). Nitrogen analyses were performed by the method of Koch and McMeekin (5), and cholesterol and hexose estimations by methods used in the Harvard pilot plant (4, 6). All colorimetric determinations were read on the Coleman universal spectrophotometer. Bioassays for PPs activity were carried out by one of us (O. W. S.) by a rat protection test such as has been described earlier (7). For each test, two litter mate male rats, 19 to 21 days old, are selected. By subcutaneous injection at the base of the tail, the control animal is given 1 cc. of normal saline, while the test animal is given the sample to be assayed dissolved in

1 cc. of saline.   30 minutes later each animal is injected subcutaneously in the nape of the neck with 1 m.l.d. of menstrual toxin in 0.5 cc. of saline.   They are then observed and the time of death noted.   The criteria for a test showing protective activity are as follows:

Positive if control dies in 18–24 hrs. and test animal lives 4 hrs. longer
   "   "   "   "   " 24–30 "   "   "   "   " 5 "   "
   "   "   "   "   " 30–36 "   "   "   "   " 6 "   "
   "   "   "   "   " 36–48 "   "   "   "   " 7 "   "

If the control animal dies before 18 or after 48 hours, the test is considered to be of no value.   The minimal protective dose of the material to be assayed is arbitrarily defined as the smallest amount which gives protection in two out of three tests.

System of Fractionation

20 liters of pooled exudate at 0° were diluted with an equal volume of cold distilled water.   The solution was then titrated to pH 5.5 by the slow addition of an acetate buffer of pH 4.0 and ionic strength 0.04.   Sufficient ethanol-water mixture was added to achieve a final volume of 70 liters (3.5 volumes), pH 5.6, ionic strength (estimated) 0.05, mole fraction of ethanol 0.062 (18 per cent by volume at 25°), at a temperature of −5°.   After 24 hours equilibration, the first precipitate (Fraction A) was separated by centrifugation.   By the addition of suitable acetate buffer and of 95 per cent ethanol, the supernatant solution was taken to 100 liters (5 volumes), pH 5.9, ionic strength 0.09, mole fraction of ethanol 0.163 (40 per cent by volume), at −5°.   Here, a second precipitate (Fraction B) was formed and similarly separated.   The solution remaining after this step was titrated to pH 5.0 by the addition of an acetate buffer of pH 4.0 and ionic strength 0.8 to effect the precipitation of Fraction C.

Fractions A and C were dried from the frozen state, whereas Fraction B was subdivided as follows.   The paste removed from the centrifuge was suspended in 12 liters of cold water and titrated to pH 5.3 by the cautious addition of an acetate buffer of pH 4.7 and ionic strength 0.1.   The estimated ionic strength of the resulting suspension was 0.006.   It was stored 24 hours at 0° and the insoluble material then centrifuged off as Fraction B-1.   The solution was taken to 20 liters at pH 5.6, ionic strength 0.02, mole fraction of ethanol 0.062 (18 per cent by volume), at −5° to precipitate Fraction B-2.   Fractions B-1 and B-2 were then dried from the frozen state as well, while the solutions remaining after the precipitation of Fractions B-2 and C were discarded.

RESULTS AND DISCUSSION

The characterization of the four fractions derived from 20 liters of exudate is given in Table I. The rejected supernatant solutions were not analyzed, but previous small scale experiments had shown them to contain little or no PPs activity, and only small amounts of protein. Fraction B-2 was submitted to electrophoretic analysis[1] and found to consist of the components shown in Table II. The results of the protection tests from

TABLE I

Characterization of Fractions of Carcinomatous Exudate

Fraction	A	B-1	B-2	C
Color	Green	Yellow	Tan	Yellow
Dry weight, gm.	350	45	42	86
Nitrogen, %	12.9	13.1	12.6	14.5
Total N, gm.	45	5.9	5.3	12.5
Cholesterol, %	3.5	0.47	0.94	0.06
Total cholesterol, gm.	12.3	0.2	0.4	0.05
Hexose, %	3.3	6.5	6.4	1.4
Total hexose, gm.	11.5	2.9	2.7	1.2
Minimal protective dose, mg.	0.25	>5	0.0125	>10
Total minimal protective dose per 100 cc. exudate	7000	<45	17,000	<43

TABLE II

Electrophoretic Components of Fraction B-2

	Albumin	α_1	α_2	β_1	β_2	γ
u × 10⁵ cm.² volt⁻¹ sec.⁻¹	6.4	5.5	4.4	3.2	2.0	1.3
% of total protein*	13	35	19	26	5	2

* Not corrected for refractive index increments.

which the minimal protective dose values were derived are given in Table III.

The conditions for the precipitation of Fraction A are a slight modification of those recently found by Cohn and his coworkers (8) to effect precipitation from human plasma of most of the components of Fractions I through IV-1 of Method 6 (4), leaving in solution some of the more soluble α- and β-globulins. Fraction B is precipitated under the conditions used for the preparation of Fraction IV-4 of Method 6, and Fraction C by the conditions used for Fraction V. The most striking difference be-

[1] We are indebted to Mr. M. Budka for carrying out the electrophoretic analysis.

tween our fractions and those of plasma is the much smaller relative amount of Fraction C than of Fraction V (these fractions representing largely albumin).

Since it was found that PPs activity tended to be destroyed at pH values of 5.0 and below, the conditions utilized in plasma work for the subfractionation of the components of Fraction IV-4 could not be employed here. It was found, however, that at low ionic strength and pH as high as 5.3 a large amount of inactive euglobulin could be removed; although,

TABLE III

Results of Rat Protection Tests with Fractions of Exudate

Fraction	Amount tested	No. of tests	Proportion showing protection	Minimal protective dose
	mg.		*per cent*	*mg.*
A	5.0	1	100	
	1.0	4	75	
	0.5	2	100	0.25
	0.25	3	67	
	0.2	4	25	
B-1	5.0	2	0	
	1.0	2	0	>5.0
	0.2	2	0	
B-2	5.0	1	100	
	0.2	2	100	
	0.1	5	100	
	0.05	3	100	0.0125
	0.025	8	87	
	0.0125	9	78	
	0.01	4	0	
C	10.0	2	0	
	5.0	2	0	>10.0
	1.0	2	0	
	0.2	2	0	

unlike Fraction IV-5, Fraction B-1 did not contain the major share of the cholesterol present in Fraction B, Fraction B-2 corresponds most closely to Fractions IV-6 + IV-7 of Surgenor *et al.* (6). We have been unable so far to separate the α- and β-globulins in the mixture without major loss of PPs activity. The close similarity between the conditions for the precipitation of Fraction A, in which PPs activity is largely soluble, and for Fraction B-2, in which PPs activity is insoluble, is noteworthy. We suggest as a basis for this change in solubility behavior the intervening simplification of the system with loss of opportunities for protein-protein interaction.

Preparation of Fraction B-2 for Trial[2]

The bulk of the dry Fraction B-2 was dissolved in physiologic saline to a protein concentration of 5 per cent and sterilized by passing through three asbestos pads in a Hormann filter. The solution was put in 5 cc. quantities into sterile bottles, frozen, dried under a vacuum, and capped sterilely. The final product was tested for sterility by culture in thioglycolate broth at 20° and 37° for 14 days, for pyrogenicity by injection intravenously into rabbits of a dose of 50 mg. per kilo, and for depressor activity by intravenous injection in anesthetized cats of a similar dose.[3] Undesirable side effects as determined in this way were at a minimum, and the material was considered satisfactory for human use. Clinical trials with the new PPs are being carried out, and will be reported in a subsequent communication from this laboratory.

SUMMARY
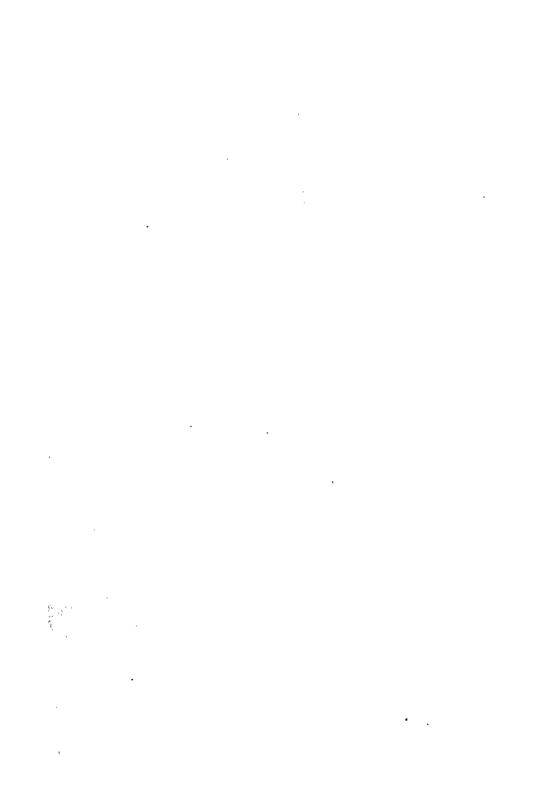
Carcinomatous exudates have been found to contain a principle, presumably a pseudoglobulin, capable of neutralizing in rats the lethal effects of a menstrual toxin. A method for the concentration of the protective pseudoglobulin is given here, yielding a product suitable for clinical trial in humans. Descriptive and analytic data concerning the active and inactive fractions obtained are reported, and the results are compared with the results of fractionation of human plasma.

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[2] We are indebted to Dr. D. Mulford and Dr. G. Edsall of the Division of Biologic Laboratories, Massachusetts Department of Health, for assistance with the sterilization, bottling, and testing for sterility and pyrogenicity.

[3] We are indebted to Dr. O. Krayer of the Department of Pharmacology, Harvard Medical School, for the tests for depressor activity.

THE REDUCTIVE CLEAVAGE OF 4-DIMETHYLAMINOAZOBEN-ZENE BY RAT LIVER: REACTIVATION OF CARBON DIOXIDE-TREATED HOMOGENATES BY RIBOFLAVIN-ADENINE DI-NUCLEOTIDE*

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High levels of dietary riboflavin greatly lower the activity of 4-dimethyl-aminoazobenzene as a hepatic carcinogen in the rat (1, 2), and the ability of various diets to inhibit the activity of this carcinogen is proportional to the level of hepatic riboflavin which these diets permit the rat to main-tain (2). Furthermore, Kensler (3) has reported that the over-all rate of destruction of the azo dye by rat liver slices is dependent upon the level of riboflavin in the hepatic tissue. These observations suggest that a functionally active form of riboflavin participates directly in one or more of the enzyme systems which determine the metabolic fate of 4-dimethyl-aminoazobenzene in the liver.

In previous communications from this laboratory (4, 5) it was reported that rat liver homogenates, fortified with glucose-6-phosphate, diphos-phopyridine nucleotide, triphosphopyridine nucleotide, and nicotinamide, catalyze the reductive cleavage of 4-dimethylaminoazobenzene to N,N-dimethyl-p-phenylenediamine and aniline. This degradation was shown to be effected by an enzyme in the particulate matter of the liver cells which requires electrons specifically from reduced triphosphopyridine nu-cleotide. The present paper deals with the participation of riboflavin-adenine dinucleotide in this reaction. It has been possible to demonstrate that much of the reductive cleavage activity is lost in hypotonic homo-genates treated with carbon dioxide and that it can be specifically regained upon the addition of riboflavin-adenine dinucleotide.

Methods

For these experiments riboflavin-adenine dinucleotide (FAD) concen-trates were isolated from hog liver essentially according to the procedure

of Sanadi and Huennekens.[1] The dry preparations reported herein ranged in purity from 14 to 61 per cent free riboflavin-adenine dinucleotide, as assayed by the fluorometric method of Bessey et al. (6). The 61 per cent preparation contained less than 1 per cent of riboflavin monophosphate (FMP) and no free riboflavin. These findings were verified by paper chromatography with butanol-acetic acid-water as the mobile phase (7). Riboflavin monophosphate solutions were prepared by the hydrolysis of riboflavin-adenine dinucleotide preparations in 0.1 N HCl for 10 minutes in a boiling water bath (6).

Glucose-6-phosphate (GL-6-PO$_4$) was prepared by the phosphorolysis of starch according to the method of Fantl and Anderson (8), but modified to include a 5 hour hydrolysis of the crude sugar phosphates in 1.0 N HCl at 100°, followed by the isolation of the ester as the barium salt. Triphosphopyridine nucleotide (TPN) concentrates were prepared by a procedure developed in this laboratory (9). These preparations ranged in purity from 45 to 85 per cent when based on the constant given by Horecker and Kornberg (10).

The liver samples were obtained from young adult albino rats[2] maintained on a grain diet and fasted for 18 to 24 hours prior to each experiment. The rats were killed by decapitation and the liver samples transferred immediately into ice-cold 1.15 per cent KCl. The homogenates (10 per cent) were prepared in 0.01 M K$_2$HPO$_4$-KH$_2$PO$_4$ (pH 7.4) at 0-3° in a Potter-Elvehjem homogenizer.

In the typical experiment a basic reaction mixture was prepared at 0° which contained the following ingredients adjusted to pH 7.4 in a final volume of 3.0 ml.: 0.3 to 0.5 ml. of homogenate, 0.4 ml. of 0.045 M glucose-6-phosphate, 0.1 ml. of diphosphopyridine nucleotide (DPN)[3] solution (200 γ), 0.2 ml. of triphosphopyridine nucleotide solution (900 γ), 0.2 ml. of 0.6 M nicotinamide, 0.1 ml. of 0.1 M MgCl$_2$, 0.4 ml. of 0.5 M KCl, and 0.5 ml. of 0.1 M K$_2$HPO$_4$-KH$_2$PO$_4$ buffer at pH 7.4. The 4-dimethylaminoazobenzene (50 to 60 γ in 0.1 ml. of 95 per cent ethanol) was added last to the ice-cold reaction mixture from a micro blow pipette. The reactions were carried out in open 25 ml. Erlenmeyer flasks with mechanical shaking in a water bath at 37°.

At the termination of the incubation period (0 to 40 minutes) the reaction was stopped by the addition of 3.0 ml. of 20 per cent trichloroacetic acid in 1:1 acetone-ethanol, and the mixture was diluted with equal parts

of this reagent and water to the appropriate optical density. The precipitated protein was sedimented by light centrifugation and the optical density of the supernatant fluid determined at 520 mμ in a Cenco-Sheard spectrophotelometer adapted to the use of matched 13 mm. $\times$ 100 mm. Pyrex culture tubes. The difference in the optical densities of the zero time control flasks and the incubated samples was used for the calculation of the total dye destruction.

The treatment of the homogenates with carbon dioxide was accomplished in two ways. When it was desirable to avoid a change in volume of the
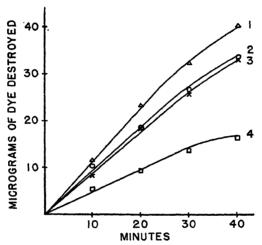
FIG. 1. The effect of additions of riboflavin-adenine dinucleotide on fresh and carbon dioxide-treated (gassed 3 hours) homogenates. Curves 1 and 2 represent fresh homogenate with and without FAD respectively; Curves 3 and 4 represent treated homogenate with and without FAD respectively. Systems as under "Methods;" the flasks contained 100 γ of FAD as indicated. Each flask contained 59 γ of dye.

homogenate, 10 to 15 ml. of the homogenate were placed in a gas washing bottle (inside diameter 6.0 cm.) packed in a beaker of chopped ice. The homogenate was then stirred slowly with a small magnetic stirrer and the air above the homogenate was replaced with carbon dioxide at atmospheric pressure. In the second method a cellophane dialysis sac (1 cm. in diameter) containing 10 to 12 ml. of homogenate was placed in a 2 liter Erlenmeyer flask containing 800 ml. of distilled water at 1–4° and a magnetic stirrer. After vigorously gassing the water for 10 minutes with carbon dioxide, the flask was stoppered and stirred. Prior to use the contents of the sac were homogenized briefly. Slightly better results and greater simplicity of operation were achieved by the latter method.

Results

Effect of Carbon Dioxide Treatment—The rate of cleavage of the dye by freshly prepared homogenates was only slightly increased by the addition of riboflavin-adenine dinucleotide concentrates (Fig. 1). However, either direct treatment with gaseous carbon dioxide or dialysis against carbon dioxide-saturated water for 1 to 3 hours resulted in a loss of 50 to 80 per cent of the reductive cleavage activity. This activity was restored nearly to the original level by the addition of riboflavin-adenine dinucleotide to
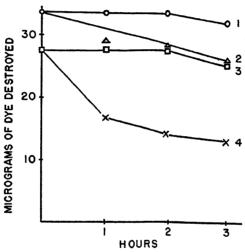
FIG. 2. The loss of cleavage activity in homogenates with and without carbon dioxide treatment (gassing for time indicated) and the effect of subsequent additions of riboflavin-adenine dinucleotide. Curves 1 and 3 represent untreated homogenate with and without FAD respectively. Curves 2 and 4 represent treated homogenate with and without FAD respectively. All homogenates were held at 0–3° before incubation. Systems as under "Methods"; the flasks contained 100 γ of FAD as indicated and 59 γ of dye; incubation time, 30 minutes.

the reaction mixture (Figs. 1 and 2). The major portion of inactivation which was reversible by the dinucleotide was obtained with 1 hour of treatment with carbon dioxide; longer exposures slowly increased the amount of inactivation which was not reversed by the coenzyme under these conditions (Fig. 2).

Specific Requirement for Riboflavin-Adenine Dinucleotide—The ability of three different preparations of the dinucleotide to reactivate a homogenate inactivated by dialysis against carbon dioxide-saturated water for 3 hours is shown in Fig. 3. All three preparations (61, 22, and 14 per cent purity) gave reactivations proportional to the amounts of riboflavin-adenine di-

nucleotide added to the reaction mixtures. In most of the experiments, 50 γ of the dinucleotide per flask were sufficient for maximal reactivation and with some homogenates only 25 γ of the cofactor were needed. This variation in response was considered to be due to variable rates of degradation of the added dinucleotide, and therefore 100 γ of riboflavin-adenine dinucleotide were customarily added when other factors were studied. In one solvent distribution experiment with a preparation of less than 10 per cent purity, the activity of the preparation was distributed between phenol and water in the same proportion as the dinucleotide.
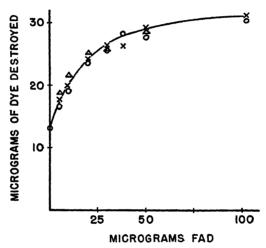
FIG. 3. The reactivation of a carbon dioxide-treated homogenate (dialysis, 2 hours) by various preparations of riboflavin-adenine dinucleotide. The preparations contained 61 per cent (O), 22 per cent (×), and 14 per cent (△) FAD. Systems as under "Methods," with 59 γ of dye per flask; incubation time, 30 minutes.

Similarly, elution tests from paper chromatograms of a crude dinucleotide concentrate with water-saturated phenol (7) or with butanol-acetic acid-water (7) showed that the activity of the crude preparation was accounted for entirely in the riboflavin-adenine dinucleotide spots, even though the R_F values of these spots were greatly different in the two solvent systems (Table I).

Evidence that the riboflavin-adenine dinucleotide concentrate was not substituting for certain other cofactor requirements is presented in Fig. 4. In this experiment the addition of the dinucleotide more than doubled the activity, while the same molar level of riboflavin monophosphate was completely inactive. Furthermore, the omission of either glucose-6-phosphate or triphosphopyridine nucleotide from the reaction mixture contain-

ing the riboflavin-adenine dinucleotide yielded a completely inactive sys
tem. The addition of 0.2 ml. of 0.01 M adenosine triphosphate (ATP)
to the complete system failed to increase the amount of dye cleavage.

TABLE I

Distribution of Active Cofactor on Paper Chromatograms of Crude.
Concentrate of Riboflavin-Adenine Dinucleotide

Systems as under "Methods," with 56 γ of dye and 0.4 ml. of homogenate dia
lyzed against carbon dioxide-saturated water for 2 hours; incubation time, 30 min
utes. The crude concentrate contained less than 10 per cent FAD; the prepara
tions added to each flask contained the equivalent of 85 γ of FAD.

Preparation added to flask	Solvent system	R_F	Dye destroyed
			γ
None...............................			17.5
Crude concentrate......................			31.8
Eluted FAD spot......................	Butanol-acetic acid-water	0.03	32.4
" " "	Phenol-water	0.7	31.9
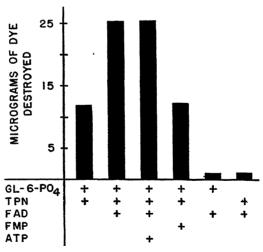
FIG. 4. The requirement for riboflavin-adenine dinucleotide by the cleavage sys·
tem in a carbon dioxide-treated homogenate (dialysis, 2 hours). Systems as under
"Methods," with 100 γ of FAD or 58 γ of FMP in the flasks indicated. Each flask
contained 59 γ of dye; incubation time, 30 minutes.

Analysis for N,N-Dimethyl-p-phenylenediamine in Homogenates Reac·
tivated with Riboflavin-Adenine Dinucleotide—For these experiments 0.4 ml
aliquots of a homogenate which had been dialyzed against carbon dioxide·

saturated water for 2 hours were added in the basic reaction mixture to each of eight Thunberg tubes. Half the samples were used as controls; to each of the other four samples, 100 γ of riboflavin-adenine dinucleotide were added. The incubation was then conducted with an atmosphere of purified nitrogen (Linde) and, after a 30 minute period, the reaction was stopped by the addition of 0.05 ml. of 50 per cent H_2SO_4.

The analysis for residual azo dyes was carried out on duplicate samples by the chromatographic procedure described earlier (4). For the determination of the accumulated N,N-dimethyl-p-phenylenediamine, duplicate acidified reaction mixtures, after the addition of 1 drop of tributyl phosphate[4] as an antifoam agent, were neutralized with solid $NaHCO_3$. The readily oxidizable amine was then trapped as a Schiff base by the addi-

TABLE II

Recovery of Metabolites of Added 4-Dimethylaminoazobenzene from Reactivated Homogenate

Systems as under "Methods," with 59.8 γ of dye and 0.4 ml. of homogenate dialyzed against carbon dioxide-saturated water for 2 hours; incubation time 30 minutes.

Amount of FAD added	Dye metabolized	Dye accounted for as		Per cent added dye accounted for
		MAB*	DMPD*	
	γ	γ	γ	
None	16.2	3.4	8.8	94
100 γ	51.1	1.3	49.8	100

* MAB, 4-monomethylaminoazobenzene; DMPD, N,N-dimethyl-p-phenylenediamine.

tion of 3.0 ml. of 0.1 per cent solution of sodium β-naphthoquinone-4-sulfonate in 1.0 M KH_2PO_4-K_2HPO_4 at pH 7.0 (5, 11). Extraction with 10.0 ml. of benzene yielded a blue-purple solution; the absorption curve of this solution had a maximum at 580 mμ and otherwise agreed with that observed for a benzene solution of the Schiff base of authentic N,N-dimethyl-p-phenylenediamine (5). The addition of acetic anhydride to the benzene solution produced only a slight decrease in optical density, which indicated that less than 10 per cent of the color could be accounted for as the easily acetylated Schiff base of N-monomethyl-p-phenylenediamine.

The results of a typical experiment are given in Table II. It can be seen that, in the absence of added riboflavin-adenine dinucleotide, only 16.2 γ of the added 4-dimethylaminoazobenzene were reduced and demethylated; of this amount 3.4 γ were accounted for as 4-monomethyl-

aminoazobenzene and 8.8 γ were accounted for as N,N-dimethyl-p-phenyl-enediamine. The addition of 100 γ of riboflavin-adenine dinucleotide increased the amount of dye that was metabolized to 51.1 γ; of this amount, only 1.3 γ could be accounted for as the monomethyl dye, while the remainder could be entirely accounted for in terms of the diamine cleavage product. In similar experiments the other cleavage product, aniline, was determined as described previously (5). The amounts of this amine that were found also accounted entirely for the dye that was destroyed. Hence under the experimental conditions employed riboflavin-adenine dinucleotide activated the reductive cleavage enzyme system but failed to increase significantly the ability of the homogenates to demethylate or hydroxylate (4) the added dye. Although all of these data were obtained with anaerobic systems, similar data were obtained under aerobic conditions, except that the recoveries of aniline, and especially of N,N-dimethyl-p-phenylenediamine, were lower (5), presumably because of oxidative losses.

DISCUSSION

Since the enzyme system mediating the reductive cleavage of 4-dimethylaminoazobenzene was largely inactivated by treatment with carbon dioxide and was subsequently reactivated by the specific addition of riboflavin-adenine dinucleotide, it seems most probable that the cleavage enzyme is a flavoprotein with this nucleotide as a prosthetic group. Attempts to apply the classical method of Warburg for the preparation of the apoenzyme of D-amino acid oxidase (12) or dialysis against HCl at pH 3.0 yielded inactive preparations which could not be reactivated. Apparently the weak acid treatment described above facilitates the dissociation of the coenzyme with very little irreversible damage to the apoenzyme. In this connection it should be mentioned that the homogenates were prepared in a hypotonic medium so as to favor the dissociation process further.[5]

It has been shown that the cleavage of the dye requires electrons from reduced triphosphopyridine nucleotide (5), and in similar experiments it has been found that the addition of cytochrome c greatly decreases the amount of dye cleavage.[6] Hence it is possible that the riboflavin-adenine dinucleotide enzyme that apparently catalyzes this reaction may normally mediate electron transport between triphosphopyridinoproteins and the cytochrome system. This possibility is supported by the finding that TPN-cytochrome c reductase is present in the microsomes (small granules) of rat liver,[6] in which the cleavage enzyme is relatively concentrated (5); this is also the site at which most of the DPN-cytochrome c reductase occurs in rat liver (13).

As the complete reduction of an azo linkage requires 4 electrons, it seems

[5] Huennekens, F. M., and Green, D. E., private communications.
[6] Mueller, G. C., and Miller, J. A., unpublished data.

likely that the reduction of 4-dimethylaminoazobenzene would proceed through the hydrazo stage and involve the transfer of two pairs of electrons. Presumably the flavoprotein mediates each step. If an intermediate hydrazo compound is formed, it does not appear to undergo readily a benzidine rearrangement at the pH employed in these enzyme systems, since in the above and previous studies (5) 100 per cent yields of either N,N-dimethyl-p-phenylenediamine or aniline respectively have been obtained in anaerobic systems. The non-enzymatic rearrangement of 4-dimethylaminohydrazobenzene to 2,4'-diamino-5-dimethylaminobiphenyl is favored by strongly acidic conditions (14). Evidence against the participation of such rearrangement products in the carcinogenic process induced by 4-dimethylaminoazobenzene and its congeners has been published recently (15, 16).

The reductive cleavage products, *viz.* N,N-dimethyl-p-phenylenediamine and aniline, have exhibited little or no carcinogenic activity in any dietary experiments devised so far (16–19). Thus it appears from the present experiments that at least part of the protective action of dietary riboflavin against carcinogenesis by 4-dimethylaminoazobenzene may consist in its participation in the cleavage of the carcinogen to relatively inactive products. However, it cannot be concluded at this time that this is the only function of riboflavin in the metabolism of this dye. A more complete evaluation of the action of this vitamin must await further investigations of the other metabolic pathways which are known to be taken by this carcinogen; *viz.*, demethylation (4, 20, 21), hydroxylation (4, 20), and the formation of protein-bound dye in the liver (11, 22).

SUMMARY

The ability of properly fortified rat liver homogenates to cleave reductively the hepatic carcinogen 4-dimethylaminoazobenzene is largely lost if the homogenates are treated with carbon dioxide at 0–3° in a hypotonic medium for 1 to 3 hours. The activity of the enzyme system is specifically regained upon the addition of riboflavin-adenine dinucleotide; as with untreated homogenates, the dye is reduced to N,N-dimethyl-p-phenylenediamine and aniline in the reconstituted system. In previous dietary experiments these amines have exhibited little or no carcinogenic activity. Hence at least part of the protective action of dietary riboflavin against carcinogenesis by 4-dimethylaminoazobenzene in the rat may consist in its participation in the cleavage of the carcinogen to relatively in active amines.

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SOME PROTEIN CHANGES IN FLUIDS OF THE DEVELOPING CHICKEN EMBRYO*

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The embryological development of the chicken egg lends itself readily to chemical study. A marked interest in this field is reflected in a vast amount of literature which has been adequately summarized by Needham (1, 2). The protein components of various embryonic fluids are particularly suited for study by the electrophoretic and ultracentrifugal techniques. Moore *et al.* (3) have previously carried out such studies of chick embryo plasma. We have undertaken to study changes in the proteins of a given embryonic fluid with the development of the embryo, as well as to attempt to find interrelationships among the protein components of such systems. In this work we have employed the blood serum, amniotic fluid, and the residual egg white proteins.

EXPERIMENTAL

Chicken eggs were incubated at 38°. The residual egg white proteins, the amniotic fluid, and the blood were collected after various periods of incubation. The blood was obtained from the vitelline vessels of the embryos and by heart puncture from the chicks.

Electrophoretic analyses were in general carried out at pH 8.6 in veronal buffer of ionic strength 0.1 in an 11 ml. cell. The protein concentration of the egg white and amniotic fluid was adjusted to approximately 2 per cent prior to analysis. The embryo serum was used undiluted because here the protein concentration ranges from 0.6 to 0.8 per cent (N × 6.25). The small amount of blood obtainable from embryos less than 10 days old made it difficult to study them by present techniques. In the electrophoresis experiments on embryo serum, the potential gradient was maintained at a value (3.0 to 3.5 volts per cm.) lower than that ordinarily used in order to prevent convective disturbances. All of the analytical measurements reported were made on the descending pattern. Mobilities were calculated from the distance between the centers of the ϵ-boundary and the peak in question.

Velocity sedimentation analyses[1] were carried out in the Svedberg oil

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[1] The sedimentation experiments were carried out by Mr. E. M. Hanson.

turbine ultracentrifuge at 185,000, $\times$ g. The Beams-Pickels preparative centrifuge was used at approximately 40,000 $\times$ g to effect a partial separation of low density lipoproteins of embryo serum.

Lipide phosphorus was determined by a combination of the methods of Youngburg (4) and Fiske and Subbarow (5). Protein nitrogen determinations were carried out by a micro-Kjeldahl procedure on samples which had been precipitated with 5 per cent trichloroacetic acid, the precipitate being washed three times with this reagent.

Results

Residual Egg White Proteins—Pools of egg white separated from eggs incubated for 14, 15, and 16 days, respectively, were studied electrophoreti-

TABLE I

Electrophoretic Analyses of Chick Embryo Serum

Age	Mobility of components $\times$ 10^5 cm.^{2} volt^{-1} sec.$^{-1}$									Per cent of components								
	Component No.									Component No.								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
days																		
10	2.9	3.7	4.9		6.2	6.9	8.1	9.0	9.9	3	1	11		9	23	39	11	2
13	2.6	3.4	4.5	5.5	6.2	7.1	8.5	9.7	10.3	5	1	12	2	13	27	25	11	4
13	2.6	3.4	4.4	5.3	6.1	6.9	8.0	9.0	9.7	7	2	12	3	10	25	29	8	3
13	2.5	3.4	4.5		6.0	7.0	8.2	9.4	10.1	4	2	12		16	32	19	10	5
13*	2.7	3.6	4.6		6.2	7.1	8.3	9.2		5	2	16		22	33	11	11	
16	2.9		4.8		6.4	7.5		8.8	10.6	6		9		7	28		42	8
19	2.7	3.9	4.5	5.3	6.1	7.3	8.4			7	2	4	8	14	42	23		
1†	2.4	3.9	4.7	5.4	6.0	6.8	7.4	8.2		14	13	10	6	8	31	16	2	
3†	2.7	3.3	4.1	5.2	5.7	6.8				14	9	17	8	9	44			
Adult female			4.2	5.0	5.8	6.9				4	5	8	6	5	36			

* Sample 3 centrifuged 4 hours in 10 per cent NaCl at 40,000 $\times$ g.

† Days from time of hatching.

cally to determine whether any change in the relative amounts of the various egg white proteins was evident. The electrophoretic analyses of all of the samples assayed were analogous to those of fresh egg white. These results indicate that there is no selective removal of any major component of the egg white proteins up to the 17th day of incubation, although a suggestion to the contrary has been made (6).

Amniotic Fluid—No appreciable amount of protein is detectable in this fluid throughout the first 13 days of incubation. By the 14th day, the sero-amniotic junction is formed between the amniotic sac and the residual egg white mass. At this time the protein concentration in the amni-

otic fluid is about 5 per cent. From the electrophoretic analyses of this fluid, taken on the 14th, 15th, and 16th days of incubation, analytical values were obtained for the egg white proteins which, within the limits of accuracy of the electrophoretic method, are identical with the values for the residual egg white.

Allantoic Fluid—In agreement with previous reports (1) this fluid was found to be essentially devoid of protein. Lyophilization of 145 ml. of dialyzed allantoic fluid from eggs incubated for 13 days yielded only 40 mg. of dry product, the major portion of which was insoluble in veronal
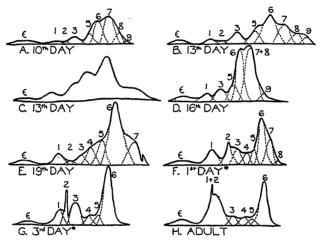
FIG. 1. Descending electrophoretic patterns of chick embryo and chicken serum. The experiments were performed in veronal buffer, pH 8.6, $\mu = 0.1$ at a potential gradient of 3 to 3.5 volts per cm., with the exception of Experiment C, which was carried out in 0.02 M sodium phosphate, 0.15 M NaCl buffer. Duration of Experiments B, C, and E through G, 300 minutes; Experiment A, 240 minutes; Experiment D, 273 minutes; Experiment H, 368 minutes. *, days after hatching.

buffer of pH 8.6. We are unable to reconcile our results with the work of Shepard and Hottle (7), who have provided electrophoretic assay data for the proteins of the allantoic fluid of the chick embryo.

Blood Serum—Typical descending electrophoretic patterns of the serum of embryos at various ages and of the serum of 1 and 3 day-old chicks and adult female chickens are shown in Fig. 1. One experiment is also recorded which was performed in a phosphate buffer at pH 7.4 of the same composition as that used by Moore *et al.* (3). The analytical values for these experiments are given in Table I. A considerable decrease in the area of Components 7 to 9 is seen as the embryo approaches maturity. By the 3rd day after hatching these components have completely disappeared.

Components 1 and 6 correspond in mobility to the γ-globulin and albumin areas, respectively, of adult chicken serum. The concentration of these proteins of lower mobility is increased 3 to 4 times in the newly hatched chick over that in 18-day-old embryos.

The electrophoretic diagram of 13 day embryo serum in phosphate-chloride buffer at pH 7.4, while showing less resolution than that obtained in the buffer at pH 8.6, is quite similar to the latter pattern. Moore *et al.* (3) made no comment on the material which has a mobility faster than that of serum albumin; *i.e.*, Components 7 to 9.

The area represented by these components showed a marked tendency toward convective disturbances. This phenomenon is probably evidence

TABLE II

Ratio of Lipide Phosphorus to Protein Nitrogen in Fractions of Chick Embryo Serum Isolated from Electrophoresis Cell

Age	Material	Nitrogen	Phos-phorus	$\dfrac{\text{Lipide P}}{\text{N}}$
days		γ *per ml.*	γ *per ml.*	
13	Whole serum	1200	68.5	0.057
	Components 7–9 on ascending side	113.5	15.5	0.137
	Through Component 5 on ascending side	457.5	41.7	0.091
	Descending side up to Component 5	213	None	
	" " " " " 6		8.5	
15	Whole serum	1070	73.0	0.068
	Components 7–9 on ascending side	193	23.7	0.123
1*	Whole serum	4025	109	0.027
	Through Component 5 on ascending side	1030	28.2	0.027
	Up to Component 5 on descending side	865	19.1	0.022

* From time of hatching.

of a relatively low density gradient and suggests that this area may contain considerable lipoprotein. Centrifugation of the embryo serum in 10 per cent NaCl, *i.e.* a medium of high density, for 3 to 4 hours in the Beams-Pickels centrifuge resulted in a fatty pellicle, high in lipide phosphorus, which formed on the surface of the serum. At the same time, there was a decrease in the area of Components 7 to 9 in the electrophoretic pattern of the serum. The analytical values are shown in Table I. Whereas Components 7, 8, and 9 comprised 34 per cent of the total embryonic serum proteins before centrifugation, they had decreased to 22 per cent after the treatment. Component 9 had actually disappeared.

That the ratio of lipide phosphorus to protein nitrogen is high in these fast components was confirmed by analyzing fractions separated by electrophoresis and removed from the cell by pipetting. (Only the ratio and

not the actual concentrations are of significance because of the dilution occurring during electrophoresis and removal of the sample.)   The results of these experiments are given in Table II.   If one assumes that all of the lipide phosphorus in the embryo serum is present as lecithin, the phospholipide concentration of the serum is 170 mg. per cent.   This is a relatively high value when the concentration of serum protein is taken into consideration.   Although the serum lipide phosphorus level is higher in the young chick than in the embryo, the chick shows a decrease in the ratio of this substance to protein nitrogen, as compared to the embryo.   The proteins of higher mobilities disappear from the serum of the chicks after hatching, and the data of Table II suggest that the lipide phosphorus is now being carried by Components 3, 4, and 5.

There is little, if any, protein phosphorus in the embryo serum.   This
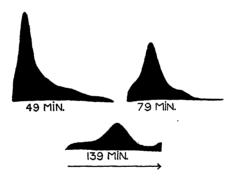
FIG. 2. Sedimentation diagrams of 13 day-old embryo serum in 0.15 M NaCl at 185,000 × *g*.

fact would appear to exclude the possibility that the faster migrating components of embryo serum are derived intact from the yolk, since the yolk protein contains large amounts of phosphoprotein (8, 9).   In a recent paper by Moore (10) a component in the electrophoretic pattern of serum of laying hens is described which has a mobility greater than that of serum albumin and which disappears on ether extraction of the serum.   Perhaps this is analogous to the components under discussion in the embryo serum.

Moore *et al.* (3) have indicated that the serum of the chick embryo is made up of a single molecular component.   Our ultracentrifugal analyses show the system to be complex.   Sedimentation patterns of serum from 13 day-old embryos at various time intervals are shown in Fig. 2.   Several areas, not clearly defined, are apparent.   The main area represents protein having a sedimentation constant of approximately 4.1 S.   The material sedimenting faster and slower than the main component will, for the sake of reference, be designated fast and slow sedimenting material.   In serum

from a 10 day-old embryo, there was relatively more of the faster sediment-
ing material and none of the slower protein. In one of the two 16 day-old
embryo pools analyzed, the slower sedimenting component was also ab-
sent; and in this case, as well as in the 10 day-old embryo serum, the main
substance sedimented more rapidly $s_{20,w} = 4.4$ S. In the serum from newly
hatched chicks there is a small amount of a component with $s_{20,w} = 7.3$ S.
The serum from the week-old chicks showed 8 per cent of a component of
sedimentation constant $s_{20,w} = 7.8$ S, which appears to correspond to the
globulins of adult chicken serum (3, 11). Even this latter sample showed
material sedimenting more slowly than the main peak, which still had a
$s_{20,w} = 4.1$ S. In adult serum the albumin has a sedimentation constant
of 4.5 S.

Velocity sedimentation experiments of 13 day-old embryo serum were
also carried out in 1 M NaCl. As expected from the results of the centri-
fugation experiments in the Beams-Pickels centrifuge, a component was
seen to sediment toward the center of rotation in this solvent. The
main peak described in the experiments performed in 0.15 M NaCl re-
solved in this case into two components of approximately equal amounts.
No attempt was made to establish any quantitative data for the sedimenta-
tion experiments carried out in 1 M NaCl solution.

SUMMARY

During the development of the chick embryo, the proteins of the egg
white and of the amniotic fluid appear to remain in the same relative
proportion in which they are present in fresh egg white. No appreciable
amount of protein was found in the allantoic fluid at any time during
incubation, in confirmation of the reports of most previous investigators.

All of the major electrophoretic components which are present in the
electrophoretic pattern of adult chicken serum appear to be present in
that of 10 day-old embryo serum, but there is a gradual change in the
relative amounts of the components as the embryo approaches maturity.
Furthermore, the patterns of the embryo serum show components with
mobilities greater than that of serum albumin. These components are
rich in phospholipide. They disappear from the serum shortly after the
chick has hatched.

Chicken embryo serum appears to contain protein components of differ-
ent molecular weight. A large proportion of these has a sedimentation
constant $s_{20,w} = 4.1$ S.

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THE TITRATABLE GROUPS OF RIBONUCLEIC ACID FROM YEAST AND OF CERTAIN FRACTIONS DERIVED BY THE ACTION OF RIBONUCLEASE

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Studies of the action of ribonuclease upon ribonucleic acid from yeast (1–5) have shown that acidic groups in the nature of secondary phosphoric acid dissociations are liberated. The phosphoester linkages which serve as a source for the secondary phosphate have not been defined. The work to be described in the following pages was undertaken in the hope of supplying such information.

EXPERIMENTAL

4 gm. of ribonucleic acid from yeast, purified according to the procedures described in a previous publication (6), were dissolved in distilled water. The solution was brought to pH 7 with alkali before adjusting to a volume of 200 ml. A 25 ml. aliquot was removed and titrated. 16 mg. of crystalline ribonuclease were added to the remaining 175 ml. of solution and a second aliquot, the control, was removed. The remaining 150 ml. and the control aliquot were incubated at 50° for 3 hours to allow action by the enzyme.

The control aliquot was stored at 5° until titrated. The 150 ml. of digested nucleic acid solution were transferred quantitatively to dialysis bags, which were only half filled. The bags were placed in distilled water in a 2 liter cylinder fitted with a stop-cock at the lower end. Dialysis proceeded in the cold room (5°). The dialysates were collected daily for a 5 day period, each analyzed colorimetrically for total phosphate (6), and thereafter lyophilized. Following completion of dialysis, the lyophilized dialysates were dissolved, mixed, lyophilized, redissolved in 200 ml. of water, and aliquots were titrated. The residue which remained in the bags was transferred quantitatively to a flask, analyzed for total phosphate, and lyophilized; the powder was dissolved and an aliquot was titrated. The control was titrated at the same time as the dialysate and residue. Boiled distilled water was used in all procedures.

The pH measurements were obtained with a Beckman pH meter, with extended shielded leads and small electrodes. The instrument was

checked frequently by calibration with phthalate (pH 4.00) and borate (pH 9.18) buffers. Titrations were performed in beakers covered with a rubber dam, a CO_2-free atmosphere being maintained over the solutions. Stirring was done magnetically. The pH readings were estimated to the nearest 0.02 unit and readings of burette delivery were made every 0.2 pH unit except in the region of pH 7 to 9 where rapid pH changes occurred. All titrations were done at room temperatures, 22–23°.

The titrations were carried out with 0.2 N solutions of hydrochloric acid and of potassium hydroxide. The use of potassium hydroxide was convenient to minimize cation corrections above pH 9 with the glass electrode. Duplicate titrations were made in every case. The deviation between points on duplicate curves was commonly less than 0.04 pH unit except in the regions below pH 2.5 and above pH 11.5.

In preliminary experiments, after titration to pH 12 with alkali, the reverse titration with acid was obtained. Such experiments were done, since Gulland and coworkers (7) observed that the back-titration of nucleic acid from thymus resulted in shifted titration curves. No significant differences in such titrations were found except in the case of the residue; the curve for pH 8 to 12 was shifted 0.5 to 0.6 pH unit to the acid side. Such a shift would indicate lowered pK' values for the enol groups present.

The residue and dialysate were recovered with great care in order that the titration values obtained might be correlated with the values for the original nucleic acid and for the hydrolyzed, unfractionated nucleic acid solution. The 150 ml. of nucleic acid solution subjected to hydrolysis and fractionation contained 229.5 mg. of phosphorus.[1] Analysis of the collected dialysate and the residue prior to its final lyophilization indicated a total phosphorus of 220.7 mg., after allowance for the aliquots removed.

The loss of 8.8 mg. of phosphorus (3.8 per cent) was ascribed to losses incidental to lyophilization since the following evidence was obtained. During the single lyophilization of the residue fraction 1.1 mg. of phosphorus were lost; during the final lyophilization of the collected dialysate 2.5 mg. of phosphorus were lost. Since the collection of dialysate involved numerous transfers and lyophilizations, the yield of dialysate was considered low by 7.7 mg. of phosphorus. With the indicated corrections, the dialyzable fraction amounted to 61.7 per cent and the residue to 38.3 per cent of the original nucleic acid phosphorus.

Results

The various titration curves obtained in a representative experiment are given in Fig. 1. Curves I and II represent the titrations of aliquots

[1] No inorganic phosphate was found in any fraction.

of the same nucleic acid solution before and after ribonuclease action, respectively. Since an inflection point in the region of pH 8.0 is a characteristic of these titration curves, all curves were brought to coincidence
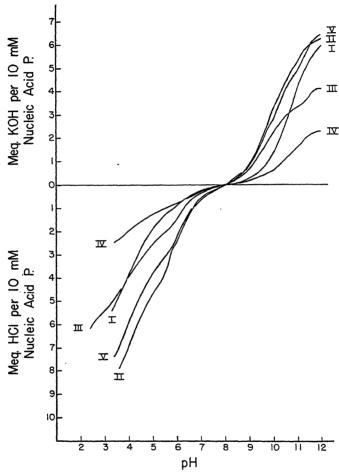
Fig. 1. The titration curves of ribose nucleic acid and derived fractions. Curve I, purified ribonucleic acid; Curve II, the ribonucleic acid following ribonuclease action; Curve III, the collected dialysate following ribonuclease action; titration data are given as milliequivalents of acid or alkali per 6.17 mM of P. Curve IV, the nondialyzable residue; titration data are given as milliequivalents of acid or alkali per 3.83 mM of P. Curve V, the arithmetic sum of Curves III and IV. The inflection points of the various curves were taken as pH 8.0 for the figure.

at pH 8.0 and 0 m.eq. of acid or alkali. This treatment was used previously by Gulland and coworkers (7). The actual inflection points obtained are given in, Table I.

For purposes of comparison with the titration curve of the original nucleic acid and the digested nucleic acid, the titration data for the di-

TABLE I

Comparison of Titratable Groups of Ribonucleic Acid from Yeast and Fractions Derived from Action of Ribonuclease

Method of calculation for various groups	Groups per 100 phosphate*						
	Fraction I	Fraction III	Fraction IV	Fraction V	Fraction II	New monoester phosphate, II − I	New Acid groups titratable to pH 8.0
Monoester phosphate							
A. Δ equivalents, pH 6.0 to inflection pH, doubled	17	47	44	46	47	30	34
B. As in A, except pK' 6.5 for Fractions I and IV	10	47	32	42	47	37	34
C. As in A; data of Allen and Eiler (1)	18				38	20	24
D. " " " " " Chantrenne *et al.* (5)	13				34	21	30
Aromatic —NH$_3^+$							
E. Δ equivalents, pH 3.5† to inflection pH less equivalent monoester phosphate by A	33	28	20	25	33		
F. As in E less equivalent monoester phosphate by B	40	28	32	29	33		
G. Sum of adenyl and cytosyl residues (Bacher and Allen (6))	49		43		49		
H. Guanyl residues (Bacher and Allen (6))	26		39		26		
Aromatic —OH							
I. Δ equivalents, inflection pH to pH 12	59	67	58	64	63		
J. Sum of guanyl and uridyl residues (Bacher and Allen (6))	51		57		51		
K. pH of inflection point (center of region of minimum slope)	7.95	7.75	8.35		7.94		

* Fractions I to V refer to the titration curves and the fractions indicated in Fig. 1.

† No complete titration of —NH$_2$ to —NH$_3^+$ groups is implied. pH 3.5 is a convenient point for comparison since it lies above the point of appearance of turbidity in the solutions.

alysate and residue have been adjusted for content of P before plotting. On the basis that 10 moles of original nucleic acid P yield 6.17 moles of

dialyzable P and 3.83 moles of residue P, the values for milliequivalents of acid or alkali per mм of P have been multiplied by 6.17 (Curve III) and 3.83 (Curve IV). The arithmetic sum of Curves III and IV yields Curve V.

Curve V has been included so that comparison may be made with Curve II, the acid-base combination of the digested nucleic acid mixture prior to fractionation. Complete coincidence of Curves II and V is expected provided that complete recovery of residue and dialyzable fragments of nucleic acid is obtained. From inspection, it is seen that reasonable coincidence was obtained in the region of pH 6 to 10. The coincidence of the curves below pH 6 is imperfect. The composition of the dialysate is changing continuously during dialysis (6); thus loss of phosphate during the fractionation may represent loss of material differing in acid-base combination according to the time when loss occurred.

DISCUSSION

It is necessary to consider (a) the interpretation of the titration curve of ribonucleic acid from yeast as concluded by others (3, 7–10) and from present data, and (b) the type and quantity of new groups titratable after ribonuclease action.

Levene and Simms (8) demonstrated that the titration curve of ribonucleic acid from yeast could be approximated in the pH region above 4.5, using their dissociation constants for the nucleotides. However, these workers could not include the expected primary phosphoryl dissociation in such curves. Fletcher and others (9) followed the titration of numerous ribonucleic acid preparations and assigned the four dissociations apparent in the region of pH 2.5 to 8.0 to three primary phosphoryl and one secondary phosphoryl group per tetranucleotide. In a later paper concerning similar titrations of thymus nucleic acid, Gulland and others (7) discarded the assumption that the primary phosphoryl dissociation of either nucleic acid was measured in the region mentioned. The region of the curve previously assigned to these groups was assigned to the dissociation of the —NH$_3^+$ groups of the guanyl, adenyl, and cytosyl radicals.

It is important to emphasize that, while the interpretation of Fletcher and coworkers (9) is at variance with the present views of Gulland and others (7), still the comparison of theoretical and practical titration curves by Fletcher et al. (9) is valid. Such a comparison is valid, since the pK′ values assigned to the primary phosphoryl groups, taken from Levene and Simms (8), were the dissociation constants of —NH$_3^+$ groups with one exception. Thus, the agreement between experimental curve and theoretical curve (see Fig. 3 of Fletcher et al. (9)) is agreement on the basis

of pK' values of 2.3, 3.7, 4.2, and 6.0.   Uridylic acid radicals were presumed absent in the theoretical mixture which gave best agreement with the experimental curve.   However, according to present concepts, uridylic acid radicals could be present in any proportion since they have no dissociable —NH_3^+ groups.

While other aspects of the titration curves of nucleic acids have been misunderstood, general agreement exists concerning the dissociation step prior to the inflection point.   This dissociation, covering the approximate region of pH 5 to 8, is assigned to monoester phosphate groups.   This region of the titration curve becomes of paramount importance in characterizing various preparations of either ribose or desoxyribose nucleic acids.   The reasons for this importance are the following: (a) the upper limit of this dissociation is the major inflection point, thus allowing accurate estimation of equivalents of acid or base at this point; (b) the region above the mid-point (pH 6 to 8) of this dissociation is free from the effects of the dissociation of other groups except the least acidic —NH_3^+ groups; (c) the molar proportion of secondary phosphoryl groups to total phosphate may be used as a criterion of structure for homogeneous preparations of nucleic acids; (d) the quantity of such residues may be used to calculate the minimum molecular weight of such preparations on the basis of certain types of phosphate linkage.

If the first and second points listed were accepted for the moment, it would be of interest to examine certain of the data of Fletcher and coworkers (9) from this view-point.   The monoester phosphate of their various preparations of purified ribose nucleic acid is 1.0 to 1.2 m.eq. per 4 mM of phosphate, values which may be compared with that of Allen and Eiler (1), 0.72 m.eq.; Gregoire (2), 0.91 m.eq.; Zittle (10) 0.82 m.eq.; Chantrenne et al. (5), 0.52 m.eq.; and that of the present work, 0.68 m.eq. The calculations were made in the same way as Calculation A for monoester phosphate in Table I.   All values are 40 per cent less with a pK' value of 6.5.

The analysis of the titration data is given in Table I.   The estimation of monoester phosphate in the various nucleic acid preparations is dependent upon the choice of pK' value assigned to this group.   The use of the mononucleotide pK' values (8) for the various groups on the polynucleotide molecule is open to the criticism that no account is taken of the interplay of charged groups at close distances in the molecule.

A pK' value of 6.5 was used for certain calculations given in Table I for the following reason.   Titration of the same nucleic acid preparation performed in the presence of added formaldehyde gave a curve which exhibited a second inflection point in the vicinity of pH 5 and 0.95 m.eq. of acid per 10 mM of phosphate when the major inflection point

was placed at 0 m.eq. This second inflection point was taken as the end of monoester phosphate titration and readings were taken of the pH corresponding to 0.47 m.eq. on every curve. These estimated pK′ values were 6.45, 6.00, 6.05, and 5.85 for titrations of (a) the control and (b) nucleic acid in 0.5 M KCl, (c) in 9 per cent formaldehyde, and (d) in formaldehyde-KCl solution respectively.[2] The first value (6.45) was used in the present calculations since the titrations were performed in the absence of added electrolyte. The location of the shift in the titration curve when formaldehyde is added is similar to that obtained with aniline by Harris (11).

It is apparent that the new monoester phosphate groups, by application of Calculation A or B of Table I, account in great part for the new acidic groups found by titration to pH 8 before and after action of the enzyme.

The agreement between new monoester phosphate and new acidic groups is important, since the latter value requires no assumed pK′ value in its calculation. Also, since the "new acidic groups" correspond to difference in titer to pH 8 before and after enzyme action, this titration has exact end-points owing to the rapid pH change in this region. Under these circumstances, 91 per cent of new groups with a pK′ value of 7 are determined, 99 per cent of groups of pK′ 6, etc. New ribose hydroxyl or nuclear hydroxyl groups would not, of course, be measured by this titration. New amino groups liberated as —NH₂ would not be determined.

Several possible types of linkage between nucleotides are listed in Table II together with the expected titratable groups after hydrolysis by ribonuclease. A structure of Type II has been postulated by Fletcher and coworkers (9) to exist in ribose nucleic acid from yeast. Such a structure, with its possibility of several monoester phosphate groups per molecule, explains the high titratable groups of this sort compared to the average molecular weight of our nucleic acid preparation (12). The hydrolysis of one linkage (Type II, a) would increase acidic groups one per bond broken but would not increase titratable monoester phosphate. The hydrolysis of the two bonds indicated (Type II, a + b) would increase both new acidic and new monoester phosphate groups. If hydrolysis according to Type II, a + b, is the reaction, monoester phosphate groups should be half the number of new acidic groups.

The pyrophosphate linkage (Type III) could account for the simultaneous appearance of monoester phosphate groups in dialysate and residue. However, such a bond is unacceptable until other modes of linkage are proved to join the dinucleotides to one another. Pyrophosphate and triester bonds (Type IV) are more acceptable since the new monoester

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[2] Duggan, E. L., unpublished data.

TABLE II
Possible Phosphoryl Linkages in Ribonucleic Acid

Type No.	Schematic formulas		Titratable groups formed by hydrolysis				
			Diester phosphate*	Nuclear —NH₂	Monoester phosphate	Nuclear —OH	Ribose —OH
I	B—R—O—P(=O)(O⁻)—O—R—B				1		1
II	B—R—O—P(=O)—O—R—B *(a)* / O—R—B *(b)*	(a)	1		1		1
		(a + b)	1†		1		2
III	B—R—O—P(=O)(O⁻)—O—P(=O)(O⁻)—O—R—B				2		
IV	B—R—O—P(=O)—O—P(=O)—O—R—B *(a)* / O⁻ O—R—B *(b)*	(a)	1		1		
		(a + b)	1†		2		1
V	R—B—O—P(=O)(O⁻)—O—R—B				1	1	
VI	R—B—O—P(=O)—O—R—B *(a)* / O—R—B *(b)*	(a)	1		1		
		(a + b)	1†		1	1	1
VII	R—B—N(H)—P(=O)(O⁻)—O—R—B			1	1		
VIII	R—B—N(H)—P(=O)—O—R—B *(a)* / O—R—B *(b)*	(a)	1	1	1		
		(a + b)	1†	1	1		1

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TABLE II—*Concluded*

B represents purine or pyrimidine radicals; R represents ribose with other ester bonds represented by vertical lines.

* Disubstituted phosphoric acid would titrate as such only after monoester phosphate and nuclear —NH_2 had been brought to —OH and $\overset{+}{NH_3}$ forms.

† Diester phosphate would be formed momentarily.

phosphate groups (Type IV, $a + b$) would be 67 per cent of the new acidic groups. A similar structure with two nucleosides on each phosphate would allow only 50 per cent agreement between the new monoester phosphate and new acidic groups if three bonds were broken during the hydrolysis. With similar reasoning, it may be concluded that structures of Types I, V, and VII are equally probable until such time as the nuclear —NH_2 and nuclear —OH groups are directly determined before and after such hydrolysis.

Perhaps the simplest explanation of the results obtained is that certain bonds of Type I are broken. In view of the analytical results obtained for the purines and pyrimidines by Bacher and Allen (6) after similar hydrolyses, it may be tentatively concluded that uridyl radicals are required in the proximity of the ester bond broken. A concomitant requirement is that the ribose nucleic acid contains regions in which guanyl radicals are concentrated and other regions in which uridyl radicals are predominant. The suggestion is tentative, since the quantity of new monoester phosphate found by titration is a function of the pK' values assigned to the group in the two previous calculations.

The monoester phosphate groups found in the dialysate fraction would agree with the composition of a mixture which contains a high proportion of nucleotides of lower molecular weight in that a high proportion of phosphate groups is titratable under the conditions used. However, the residue fraction has maintained an average molecular weight of several thousand (12) while showing titratable phosphate groups in the proportion of one group per three phosphates.

The titration of the —NH_2 groups of the adenyl, cytosyl, and guanyl radicals is incomplete at pH 3.5. This pH was taken as a comparison point for all fractions, since several of the fractions became turbid on the further addition of acid. The data in Table I, Calculations F, G, and H, indicate that 75 to 80 per cent of the adenyl and cytidyl amino groups have become charged at pH 3.5. It is interesting that the increase in guanyl radicals in the residue fraction (Curve IV) is not reflected in increased uptake of hydrogen ion at this pH. This fact is reasonably explained on the basis that the amino group of guanosine is a 100-fold weaker as a base than similar groups of adenosine and cytidine (8).

While great changes have occurred in the quantity of monoester phos-

phate groups, slight changes are shown in the quantity of enol groups. From the position of the titration curves on Fig. 1, it is apparent that there is a shift in the pK′ value of the enol groups in the cases of the dialysate (Curve III) and summation (Curve II or V), but no corresponding shift is apparent in the case of the residue fraction (Curve IV). In the pH region above the inflection point, Curves III and II are similar when Curve III is plotted for dissociation per 10 mM of phosphate; Curves IV and I are identical in shape when both are plotted on this basis.

The comparison of titratable groups for this alkaline region with the number found by analysis indicates that a greater number of groups appears by titration. There is no sign that the titration is complete at pH 12 from the shape of the curves obtained. The agreement between titration and analytical values for the residue (Curve IV) may be fortuitous, since this curve is displaced 0.5 pH unit to the alkaline side of the other curves at the inflection point.

SUMMARY

1. The titration curves of ribonucleic acid from yeast, ribonuclease-hydrolyzed nucleic acid, a dialyzable fraction, and a residue fraction have been obtained.

2. Evidence is presented for the view that the titration curve of ribonucleic acid from yeast (pH 3 to 12) represents the dissociation in turn of nuclear $—NH_3^+$ groups, monoester phosphate groups, and nuclear hydroxyl (enol) groups.

3. The new titratable groups following the fragmentation of this ribonucleic acid by crystalline ribonuclease were found to be 34 m.eq. per 100 mM of phosphate. With pK′ values of 6.0 or of 6.5 for the monoester phosphate group, 30 or 37 m.eq. of new groups per 100 mM of phosphate were found. On the basis of such approximate agreement, it would seem that the result of the breaking of bonds by the action of ribonuclease is the formation of a sugar hydroxyl group and a monoester phosphate group. Reasons are given for the choice of pK′ value for the group.

4. Calculations of the ratios of monoester phosphate to total phosphate in the original nucleic acid, the dialysate, and the residue give the values of 1:10, 1:2, and 1:3, respectively. Thus, appreciable quantities of new groups have appeared in the residue as well as in the dialysate.

5. The number of titratable amino groups varies in the fractions but is about 80 per cent of the analytical values for adenine and cytosine in the preparations used.

6. The number of enol groups obtained by titration from the inflection pH to pH 12 was found to be slightly greater than the number of such groups from analysis. The titration curves in this range indicate a de-

crease in the enol pK' value of about 0.5 pH unit in the case of the dialysate fraction or of the dialysate-residue mixture.

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INFLUENCE OF THYROID ACTIVITY ON EXCHANGE OF LIVER ADENOSINE TRIPHOSPHATE PHOSPHORUS*

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The utilization of the energy of the oxidative processes of the Krebs tricarboxylic acid cycle for the synthesis of compounds with high energy phosphate bonds (adenosine triphosphate (ATP) or creatine phosphate) has been demonstrated *in vitro* with tissue extracts, slices, and homogenates (1–7). Furthermore, it might be expected that if oxidative reactions were accelerated in the intact rat an increased rate of ATP synthesis would occur. In view of the foregoing, it was considered of interest to investigate the synthesis of a compound with high energy phosphate bonds, *viz.* ATP, at different levels of thyroid activity, the normal, hypo-, and hyperthyroid states.

Kaplan and Greenberg (8) have shown that the administration of insulin *in vivo* is associated with an increase in the synthesis of liver ATP.

EXPERIMENTAL

Animals; Care and Treatment—Male rats of the Long-Evans strain, weighing between 200 and 250 gm. before experimentation, were used. The three groups each consisted of eight to ten rats. The control group was maintained on the stock diet, while the hyperthyroid group received the stock diet containing 1 per cent desiccated thyroid (Armour) for 21 to 25 days (9). Hypothyroidism was induced in the third group by including 0.2 per cent thiouracil (deracil, Lederle) in the stock diet for 3 weeks. This diet has been shown by Barker (10) to produce a state of hypothyroidism with a decrease of about 20 per cent in the metabolic rate. The three groups of animals received food and water *ad libitum* and were fasted for 16 to 18 hours before sacrifice. The rats were anesthetized with nembutal (80 mg. per kilo) injected intraperitoneally, except that in the case of the hyperthyroid animals only half the dose of the anesthetic was administered. All animals were injected via the external jugular vein

with the equivalent of 6 to 7 $\times$ 10^6 c.p.m. of radioactive disodium phosphate in normal saline. The total amount of phosphate administered was physiologically negligible, being less than 0.2 mg. After 5 minutes, a sample of blood was drawn from the inferior vena cava, the liver rapidly excised and plunged into a dry ice-acetone bath.

Preparation of Trichloroacetic Acid (TCA) Extracts—The frozen liver tissue, after being weighed, was transferred to a prechilled steel mortar containing powdered dry ice moistened with acetone and was broken into small particles. It was then transferred to a piece of wax paper and allowed to remain until most of the dry ice had evaporated. Then the sample was ground in a chilled Potter-Elvehjem (11) homogenizer with about 20 ml. of 7 per cent TCA and centrifuged in a 50 ml. plastic tube at 4500 r.p.m. for 2 minutes in a refrigerated centrifuge. The supernatant was poured into a 50 ml. volumetric flask and the residue reextracted with an additional 20 ml. portion of 7 per cent TCA which has been used to wash down the homogenizer. After centrifuging, the combined TCA extracts were made up to volume and kept at 0°.

Analysis of TCA Extract[1]—Immediately after the preparation of the extract, 1 ml. aliquots were neutralized with 4 ml. of 0.1 M sodium acetate and made up to a 25 ml. volume with acetate buffer of pH 4.2 (6). From this, 10 ml. aliquots were used for the determination of inorganic phosphate by the method of Lowry and Lopez (13), readings being made with a Klett-Summerson colorimeter.

3 ml. aliquots of TCA extract were neutralized with 10 per cent ammonia and inorganic phosphorus was precipitated[2] as magnesium ammonium phosphate with the magnesia mixture described by Sacks and Sacks, as mentioned by Cori and Cori (14). Carrier phosphate was added in all experiments which involved the precipitation of radioactive inorganic phosphorus. The mixture was allowed to stand for 6 to 8 hours in a refrigerator and centrifuged, and the supernatant carefully removed. The precipitate was washed twice with 5 to 8 ml. of 2.5 per cent ammonia and dissolved in dilute hydrochloric acid and made up to a suitable volume. Aliquots were plated in ointment capsules and evaporated to dryness at 60° after mixing with 1 ml. of a solution of a mixture of 1 per cent Nacconol (a synthetic detergent) and 1 per cent disodium hydrogen phosphate to insure uniform spreading. The capsules were greased on the inside to prevent creeping of the samples on the walls. Radioactivity was measured with an autoscaler model Geiger-Müller counter, the counting rate being usually 10 to 20 times the background.

[1] For a recent fractionation procedure for the acid-soluble phosphorus compounds of liver modified from Kaplan and Greenberg, see Sacks (12).

[2] For the precipitation of inorganic P[32] from various fractions of the acid-soluble phosphates, the precautions outlined by Kaplan and Greenberg (8) were followed.

Total radioactive phosphate (P^{32}) in the TCA extract was measured after evaporation of a suitably diluted sample of an aliquot of the extract. Total P^{31} estimations were carried out by the method of Fiske and Subbarow (15) as modified by LePage and Umbreit (16).

Preparation.of Mercuric-Insoluble Nucleotides—A 35 ml. portion of the TCA extract was carefully neutralized to methyl red, first with 10 N and then with 0.5 N sodium hydroxide (silicate-free) in an ice bath. The nucleotides were precipitated by the addition of a solution of 20 per cent mercuric acetate in 2 per cent acetic acid according to Kerr (17). Under these conditions practically no inorganic phosphate is precipitated. The mixture was placed in the refrigerator for 6 to 8 hours and the precipitate collected by spinning in a refrigerated centrifuge at 2400 r.p.m. for 30 minutes. The supernatant was discarded and the residue washed once with 6 ml. of ice-cold diluted (1:40) mercuric acetate-acetic acid solution. After this procedure the precipitate was usually left overnight at $-10°$. The mercury was removed by suspending the salt in 10 ml. of ice-cold water and passing hydrogen sulfide through the suspension in the cold bath for 30 minutes. Mercuric sulfide was removed by centrifugation and washed with 5 ml. of cold water. The combined extracts were aerated in the cold for 2 hours to remove the H_2S, neutralized to pH 7.4 to 7.6 (brom thymol blue), and made up to 25 ml. In order to exclude any possibility of contamination of the organic radioactive phosphate with the strongly radioactive inorganic phosphate, the mercury nucleotide fraction was subjected to a "washing" with inert phosphate as described by Kalckar, Dehlinger, and Mehler (18). This procedure was followed only in the initial stages of our investigation as a check on the experimental technique.

The total P^{31} and P^{32} content of the nucleotides was determined as described above.

7 Minute-Hydrolyzable Phosphorus—A mixture of 2 ml. aliquots of the nucleotide fraction, 3 ml. of water, and 1 ml. of 6 N hydrochloric acid was hydrolyzed for 7 minutes in a boiling water bath. The volume was then made up to 10 ml. 2 ml. aliquots were used for P^{31} estimations (15). A 5 ml. aliquot was used to precipitate the inorganic phosphate and to estimate the labile P^{32}.

Determination of ATP in Mixture of Nucleotides—The procedure was based on the observation that purified myosin acts as an ATPase, converting ATP into adenosine diphosphate with the liberation of 1 mole of inorganic P^{31} per mole of ATP. The myosin used in these experiments was prepared according to Bailey (19) from rabbit muscle. The final product was lyophilized and stored at $0°$. A sample of myosin was considered useful for ATP assay if it was free from inorganic P and diphosphatase activity (20). The preparation was assayed against ATP by the semimicromethod of DuBois and Potter (21). The sample of myosin

prepared by us liberated 40 γ of labile phosphorus per mg. of protein in 15 minutes at 37°.

For the determination of ATP in mercuric insoluble nucleotides, the reaction mixture consisted of 1 ml. of myosin homogenate containing 5 mg. of protein in 0.1 M veronal buffer (Michaelis) at pH 7.4 to 7.5, 0.08 ml. of 0.1 M calcium chloride, and 2 ml. of the nucleotide solution. After incubation for 15 minutes at 37°, the vessels were chilled, and 1 ml. of 20 per cent cold TCA added. The volume was made up to 5 ml. with ice-cold water and the samples centrifuged. 1 ml. samples of the clear centrifugate were neutralized with 4 ml. of 0.1 M sodium acetate and diluted to 10 ml. with acetate buffer (pH 4.2) (6), and the inorganic phosphorus

TABLE I

Recovery of Adenosine Triphosphate Added to Trichloroacetic Acid Extracts

For each experiment, the liver from two rats was pooled and the TCA extract made up to 100 ml. To an aliquot of 35 ml. of TCA extract, the tetrasodium salt of ATP trihydrate* equivalent to the 7 minute-labile P indicated was added and the procedure outlined in the text was followed. A second aliquot of 35 ml. was worked up with no additions. The recovery values are the mean of duplicate assays on the nucleotide fractions.

7 min.-labile P added	P recovered			
	7 min.-labile, increase		Terminal labile (enzymatic), increase	
γ	γ	*per cent*	γ	*per cent*
1000	966	97	470	94
160	153	96	74	92
80	75	94	36	90

* ATP·Na$_4$·3H$_2$O, Rohm and Haas Company, Philadelphia; contained 1.4 per cent inorganic phosphate; ratio of labile P to total P, 0.67.

determined (13). The P^{32} in the reaction mixture was determined in the inorganic phosphate precipitated from a 3 ml. sample.

Suitable control samples were run to determine the enzyme blank, reagent blank, and spontaneous breakdown of ATP. The inorganic phosphorus liberated during incubation represents one-third of the ATP phosphorus content of the substrate.

Preliminary experiments designed to study the recovery of ATP added to TCA extract of liver following mercuric precipitation and assay by myosin showed a recovery of 90 to 94 per cent (Table I).

Plasma Inorganic P^{31} and P^{32}—2 ml. plasma samples were extracted with 7 per cent TCA and made up to 15 ml. 1 ml. aliquots were used for P estimation by the method of Lowry and Lopez (13) and 5 ml. samples for the determination of inorganic P^{32} after precipitation with magnesia mixture.

DISCUSSION

The results in Table II show that there is no appreciable difference in the P^{31} values of the plasma and of the various fractions of the TCA-soluble extract of the liver among the three groups of rats.   These values

TABLE II

Distribution of P^{31} and P^{32} in Acid-Soluble Phosphate of Liver and Plasma Five Minutes after Intravenous Administration of $Na_2HP^{32}O_4$

	Control, 10 rats	Hyperthyroid, 8 rats	Hypothyroid, 8 rats
Plasma			
Inorganic P^{31}......................	7.0 ± 0.3	6.2 ± 0.2	7.4 ± 0.8
" P^{32}......................	106.3 ± 4.3	71.7 ± 3.6	164.4 ± 11.8
SA................................	151.8 ± 5.5	116.8 ± 8.3	224.2 ± 15.9
Acid-soluble liver extract			
Total P^{31}..........................	73.2 ± 2.4	74.1 ± 1.6	74.0 ± 1.8
" P^{32}..........................	90.2 ± 6.7	144.6 ± 6.1	70.9 ± 3.2
SA................................	12.4 ± 0.7	20.1 ± 1.6	9.7 ± 0.7
Inorganic P^{31}......................	23.4 ± 0.9	23.4 ± 0.8	22.1 ± 0.8
" P^{32}......................	59.9 ± 2.7	76.0 ± 5.4	50.7 ± 2.6
SA................................	25.5 ± 1.9	32.7 ± 2.2	23.0 ± 0.8
Mercuric insoluble nucleotides			
Total P^{31}..........................	31.8 ± 2.0	27.4 ± 1.0	28.1 ± 1.7
" P^{32}..........................	32.6 ± 2.9	67.6 ± 3.8	21.2 ± 2.8
SA................................	10.2 ± 0.2	24.7 ± 1.4	7.5 ± 0.7
7 min.-hydrolyzable P^{31}............	13.0 ± 0.3	12.9 ± 0.6	11.2 ± 0.7
7 " P^{32}..........	30.1 ± 2.6	65.5 ± 3.7	19.9 ± 1.2
SA................................	23.3 ± 1.9	51.4 ± 3.7	18.8 ± 0.9
Terminal labile P^{31} of ATP........	2.4 ± 0.1	2.2 ± 0.2	2.3 ± 0.1
" " P^{32} " "	29.1 ± 2.3	63.9 ± 3.5	18.6 ± 1.2
SA................................	122.5 ± 7.8	295.0 ± 16.2	85.2 ± 4.6

The P^{31} values are in mg. per 100 gm. of fresh liver or 100 ml. of plasma.   The P^{32} values are in parts per 100 of the administered dose per 100 gm. of fresh liver or 100 ml. of plasma.   The specific activity (SA) is expressed as $P^{32}/P^{31} \times 10$.   The measure of variability is the standard error of the mean.

are lower than those reported by Kaplan and Greenberg (8); the differences may be due to the shorter time that the TCA remained in contact with the tissues in the present procedure (22), indicating less hydrolysis of labile P compounds by the reagents.   The terminal labile P^{31} values of ATP in Table II agree well with Rapoport's results (23) for fasting rats.

The 7 minute-hydrolyzable P^{31} values are, of course, several times greater than the terminal labile P^{31} of ATP, since the mercuric-insoluble nucleotides, which consist mostly of ADP and less so of ATP, yield two-thirds of the phosphorus of ATP and one-half the ADP phosphorus on brisk acid hydrolysis.

However, the differences in P^{32} values are significant for the hypo- and hyperthyroid animals. The specific activity (SA) of inorganic phosphorus in plasma for the hyperthyroid animals is less (-23 per cent) than the control; the reverse situation exists for the hypothyroid animals ($+48$ per cent). The terminal P of ATP and the other liver fractions studied show the same feature of high P^{32} values (and consequently higher SA values, since the P^{31} values do not differ for the three groups of rats) for the hyperthyroid group, and smaller values for the hypothyroid animals.

But it is important to stress that the relative amounts of P^{32} recovered in the ATP are not necessarily a measure of the relative rates of turnover. The turnover cannot be judged by the rate of exchange with the administered inorganic P^{32} in this instance, since the rate of transfer of the P^{32} from the plasma to the liver differs markedly in the three groups of rats. The amount of P^{32} that the ATP will exchange depends on the specific activity of the P^{32} within the cell, where the exchange process takes place. If the phosphate with which ATP was exchanging has a higher SA in the hyperthyroid than in the hypothyroid, there would be more P^{32} incorporated, even if the turnover was the same in the two cases.

Furthermore the SA of the terminal group of the ATP is higher than the SA of any fractions determined in the liver and furthermore it is higher than the inorganic P of the plasma in the hyperthyroid group. Since this is the case, we are unable to reach a valid conclusion about the turnover of the ATP, because the SA of the P precursor with which ATP reacts is unknown. It is doubtful that any phosphorus compounds, other than inorganic P, participate in the dynamic exchange with ATP, since the total amount of P^{32} in the liver is satisfactorily accounted for in all groups of rats by the sum of inorganic P^{32} plus terminal P^{32} of ATP. However, the SA of inorganic P in liver is not valid for turnover calculations, since this value is the sum of intracellular and extracellular inorganic phosphate.

It must be pointed out that a differentiation between the extracellular and intracellular inorganic phosphate would help to arrive at a quantitative evaluation of the rates of turnover of the phosphorylated compound, but it is not possible to satisfy this distinction experimentally. It is well known that, in a short time experiment, the plasma P^{32} does not equilibrate with the extracellular space (24) and therefore any evaluation of the intracellular inorganic P^{32} on the basis of extracellular space of the tissue is

not valid.   Perfusion of the tissue under ice-cold conditions to eliminate the extracellular phosphate is objectionable on the score that anoxia and low temperature can hardly be expected to leave the permeability of the cell membrane unaffected (25).   Perfusion at body temperature will result in rapid breakdown of ATP and consequently obscure the findings.   However, Kalckar *et al.* (18) are inclined to believe that the correction for extracellular phosphate in the liver is small because of the rapid penetration of the phosphate into the liver cells.

The observation that labeled inorganic phosphate disappeared from the plasma faster in the case of the hyperthyroid rat than in the control and the plasma P^{32} is greater in the control than in the hypothyroid confirms previous findings (26).   It was demonstrated that, at the end of 110 minutes after intraperitoneal administration of a tracer dose of radioactive Na_2HPO_4, the serum inorganic P^{32} was decreased and P^{32} content of the total acid-soluble, inorganic, and labile P fractions of muscle was 100 per cent greater in the hyperthyroid than in the fasted controls.   In hypo-. thyroidism the level of inorganic P^{32} was increased in blood serum and the muscle showed the lowest ability to concentrate phosphorus.   From these observations, it was suggested that thyroid activity may influence the rate of transfer of phosphorus across cell membranes.   That a chemical mode of transfer of P from the extracellular space to the interior of the cell may operate had been suggested earlier by Sacks and Altshuler (27) and gained further support from the work of Kamen and Spiegelman (28) on phosphate metabolism in unicellular organisms and a number of other similar investigations on cell suspensions (29–31).

Whether the cell membrane is the factor responsible for the accelerated transfer of P^{32} into the cell in hyperthyroidism or not is difficult to decide. Indeed, it is entirely possible that accelerated or retarded phosphorylation steps of the glycolytic cycle under altered thyroid conditions may explain the difference in the P^{32} concentration of plasma and liver inorganic phosphate in the three groups.   Further work is necessary to decide between these alternatives.

SUMMARY

1. Labeled phosphate in the form of Na_2HPO_4 was administered intravenously to normal, hyper-, and hypothyroid rats in a postabsorptive state. P^{31} and P^{32} in the acid-soluble phosphorus fractions of the liver and plasma have been determined.

2. The ATP of TCA extract of liver, precipitated together with other mercuric-insoluble nucleotides, has been determined by the use of adenosinetriphosphatase (myosin) prepared from rabbit muscle.

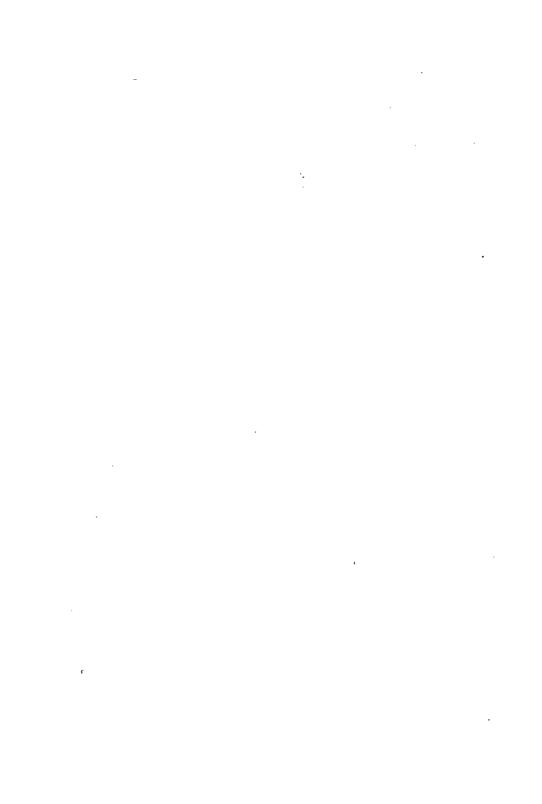3. While there appears to be no marked variation in the P^{31} values of

the acid-soluble fractions of the livers between normal, hyper-, and hypo-thyroid rats, the differences in P^{32} values are significant.

4. In a short time experiment, the organic binding of P^{32} in liver is mostly due to its incorporation into the terminal group of ATP.

5. The P^{32} concentrations of plasma inorganic phosphate in normal, hyper-, and hypothyroid animals indicate an accelerated transfer of P^{32} from plasma to cell in hyperthyroidism and a retarded transfer in hypo-thyroidism.

6. The different specific activities of the terminal labile P of ATP in the hyper- and hypothyroid groups may reflect accelerated and retarded oxidations, respectively, within the cell.

7. It has not been possible to decide whether an increased *rate of synthesis* of ATP occurs or not, since the specific activity of the intracellular inorganic P is not known.

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BIOCHEMICAL STABILITY OF THE METHYL GROUP
OF CREATINE AND CREATININE*

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Recent investigations in this laboratory (1–3) have shown that appreciable quantities of the methyl group ingested in the form of methionine are oxidized to carbon dioxide. Inasmuch as the methyl groups of methionine, choline, and betaine are directly or indirectly interchangeable in the animal body, it is evident that the methyl groups supplied by choline and betaine, indeed by all "biologically labile" methyl compounds, can likewise be degraded to carbon dioxide and water. However, the experiments with methionine provide no information concerning the oxidation of the methyl group of creatine, since the methyl group of creatine does not participate in transmethylation reactions (4). A *direct* test of the susceptibility of this methyl group to oxidation was therefore desirable. Creatine and creatinine containing C^{14} in the methyl group were synthesized and administered to rats in doses of 1.1 to 1.4 gm. per kilo of body weight. The expired carbon dioxide was collected and examined for the presence of radioactivity. The amount of C^{14} excreted in the urine and feces was also determined.

EXPERIMENTAL

Synthesis of Radiocreatine—Barium carbonate[1] containing C^{14} was converted to radiomethyl iodide by the method of Melville, Rachele, and Keller (5). The methyl iodide was then condensed with p-toluenesulfonylglycine to yield p-toluenesulfonylsarcosine (6). The latter compound was hydrolyzed with concentrated hydrochloric acid and the sarcosine, labeled with C^{14} in the methyl group, was isolated as the hydrochloride.[2]

500 mg. of the radioactive sarcosine hydrochloride were dissolved in 1.6 ml. of water. 0.45 ml. of concentrated ammonium hydroxide and 1.2 ml.

[1] The radioactive barium carbonate used in this investigation was supplied by the Monsanto Chemical Company, on allocation from the Isotopes Division, United States Atomic Energy Commission.

[2] We wish to thank Dr. Donald B. Melville for the synthesis of radiomethyl iodide and for his collaboration in the synthesis of radiosarcosine.

of a 30 per cent solution of cyanamide were then added, and the combined solutions were allowed to stand at room temperature. Crystals of creatine hydrate appeared on the 2nd day. After 3 days the reaction mixture was placed in the refrigerator for 2 more days. At the end of this time it was chilled in an ice bath and the supernatant was removed by aspiration. The crystals of creatine hydrate were washed 5 times with small portions of ice-cold water, and then suspended in ice-cold ethanol and transferred to a filter. They were washed on the filter with ether and dried at the water pump.

The creatine hydrate was converted to creatine by heating at 105°. The loss in weight corresponded to the theoretical value for the water of hydration. The purity of the product was demonstrated by the Jaffe reaction, which gave a creatine content of 100.2 per cent. The yield of radiocreatine from the radiosarcosine hydrochloride was 65 per cent.

1 mg. of the creatine gave 6.66 $\times$ 10^4 c.p.m. after correction for background and self-absorption. A portion of the creatine was converted to creatinine by boiling the solution down to near dryness in dilute hydrochloric acid as described by Benedict (7).

Metabolism Experiments—The young male rats used in these experiments were obtained from Rockland Farms. Approximately 10 days prior to receiving radioactive creatine or creatinine, the animals were placed on Diet I, a purified amino acid diet containing 1.2 per cent methionine (3). Immediately following the administration of a test compound, each rat was placed in a metabolism chamber and the expired carbon dioxide, urine, and feces were collected for the next 24 or 54 hours. During this time the animals were given free access to the diet. The metabolism apparatus and the methods employed for measuring the C^{14} content of the carbon dioxide, urine, and feces have been described in a previous publication (2).

Unless otherwise stated, the carbon dioxide expired on the 1st day was collected in periods of 4, 4, and 16 hours. An aliquot taken from the solution collected in each period was converted to barium carbonate and counted in a thin mica window Geiger-Müller counter. The barium carbonate sample and the background were counted four times in rotation until a total of approximately 1000 counts had been obtained on each.

In the first experiment 21.1 mg. of radiocreatine, plus 2 gm. of the diet, were administered to a 143 gm. rat by stomach tube. The samples of barium carbonate prepared from the carbon dioxide expired during the three collection periods gave counts that did not differ significantly from the background count.

The next rat was given 21.2 mg. of radiocreatine by intraperitoneal injection. At the same time 2 gm. of the diet were given by stomach tube. In this experiment the expired carbon dioxide was collected at hourly in-

tervals for the first 4 hours and at 2 hour intervals for the next 4 hours. Thereafter, collections were made at the end of 4, 12, 6, and 24 hour periods to give a total collection time of 54 hours. The average difference in counts per minute between a sample and its background was ±1.6; the extremes were −4 and +3 c.p.m. More than one-half of the samples gave counts below those of their respective backgrounds.

In an attempt to provide conditions favoring the oxidation of the methyl group of creatine, a third rat was placed on Diet I plus 8 per cent of non-isotopic creatine for 8 days. The average food consumption was 10 gm. per day. On the 9th day 20.9 mg. of radiocreatine were injected intraperitoneally. During the next 24 hours 8 gm. of the creatine diet were consumed. In this experiment, the barium carbonate prepared from an aliquot of the carbon dioxide collected during the first 4 hours was counted for a longer period of time. 2000 counts were obtained on both the sample and the background on four separate occasions. Each time the sample counted slightly higher than the background, giving an average of 0.8 c.p.m. above the background. When corrected for self-absorption and the size of the carbon dioxide aliquot employed, this level of radioactivity corresponded to the C^{14} present in the methyl group of 9 γ, or 0.04 per cent, of the injected creatine. The barium carbonate samples prepared from the carbon dioxide collected in the following 4 and 16 hour periods gave 0.1 c.p.m. above the background.

The susceptibility of the methyl group of creatinine to oxidation was next investigated. A rat previously maintained on Diet I was injected intraperitoneally with 18.5 mg. of radiocreatinine, a dose equivalent to 21.4 mg. of creatine. The carbon dioxide expired during the first 4 hours contained C^{14} equivalent to 28 γ, or 0.15 per cent, of the injected creatinine. Radioactivity was not detected in the carbon dioxide collected during the remainder of the experiment.

Aliquots of the urine and feces collected in each of these experiments were dried over phosphorus pentoxide, and wet ashed by the procedure of Van Slyke and Folch (8). The evolved carbon dioxide was collected in alkali and its C^{14} content was determined. Since the methyl group of creatine and creatinine does not participate in transmethylation reactions (4) and since we have been unable to detect appreciable oxidation, it is reasonable to assume that the excreted methyl groups were still part of the administered molecule or a derivative thereof. The biochemical stability of the nitrogen portion of the creatine molecule has been demonstrated by Bloch, Schoenheimer, and Rittenberg (9).

As is shown in Table I, 15 per cent of the radiomethyl groups administered as creatine by stomach tube was excreted in the urine in 24 hours. The injection of a comparable dose nearly doubled the percentage ex-

creted. However, when a rat was maintained for 8 days on a diet containing 8 per cent of creatine (non-isotopic), the injection of radiocreatine resulted in the excretion of 95 per cent of the administered methyl groups in 24 hours. This observation is of interest in view of the fact that a complete balance has never been obtained following the administration of creatine (10–14).

TABLE I

Excretion in 24 Hours of Methyl Group of Exogenous Creatine and Creatinine Labeled With C^{14} in Methyl Radical

Diet	Weight of rat	Treatment	Per cent of administered methyl group excreted	
			Urine	Feces
	gm.			
Diet I*	143	21.1 mg. radiocreatine orally	15.1	0.63
" "	157	21.2 " " intraperitoneally	27.6†	0.28†
" " + 8% creatine	190	20.9 mg. radiocreatine intraperitoneally	94.7	0.01
" "	159	18.5 mg. radiocreatinine intraperitoneally	81.5	0.13

* A purified amino acid diet containing 1.2 per cent methionine (3).

† During the next 30 hours 1.84 per cent was excreted in the urine and 0.08 per cent in the feces.

DISCUSSION

The experiments described in this paper demonstrate that the methyl group of creatine and creatinine is not oxidized to any appreciable extent by the rat. In this respect the methyl group of creatine and creatinine occupies a special category among the methyl groups thus far investigated. Under identical experimental conditions the methyl group administered as an equimolar amount of methionine was rapidly oxidized, 25 per cent of an oral dose and 31 per cent of an injected dose being converted to carbon dioxide and water in 24 hours (3). Similarly, we have found that 50 per cent of the methyl group injected as an equimolar amount of sarcosine was oxidized in 24 hours.[3]

It is obvious from the results with methionine that those methyl groups which can be transferred directly or indirectly in the body to methionine, such as the methyl groups of choline and betaine, can also be oxidized. However, the ability of a methyl group to enter into transmethylation reactions is not a prerequisite for oxidation. It has recently been observed

[3] Mackenzie, C. G., and du Vigneaud, V., unpublished work.

that the methyl groups of p-dimethylaminoazobenzene were oxidized under conditions in which transmethylation could not be detected (15). Consequently, the resistance of the methyl group of creatine and creatinine to oxidation cannot be attributed solely to its non-labile nature.

SUMMARY

Creatine and creatinine labeled with C^{14} in the methyl group have been synthesized and administered to rats in single oral or parenteral doses of 1.1 to 1.4 gm. per kilo of body weight. The expired carbon dioxide collected during the next 1 or 2 days contained little or none of the labeled carbon. It may be concluded therefore that, under the experimental conditions employed, the methyl group of creatine and creatinine is not oxidized to an appreciable extent by the intact rat. In this respect creatine and creatinine differ from the other methyl compounds so far investigated.

The excretion of C^{14} in the urine and feces following the administration of radiocreatine or radiocreatinine is reported.

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OXIDATION OF ISOTOPIC PALMITIC ACID IN ANIMAL TISSUES*

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The present study of the oxidation, by surviving tissues, of the natural 16-carbon fatty acid, palmitic acid, was undertaken to supplement previous studies of the oxidation *in vitro* of short chain fatty acids ranging from 2 to 10 carbon atoms (1–5). Though these studies of the short chain acids have gone far in clarifying many aspects of fatty acid oxidation, more direct information concerning the catabolism of the natural long chain acids seemed desirable, inasmuch as this process is recognized to represent one of the major metabolic activities of animal cells.

The first attempt to study the breakdown of the longer chain acids *in vitro* was made by Muñoz and Leloir (6) who found that, whereas acids of up to 8 carbon atoms caused definite increases in the oxygen consumption of a suitably fortified, washed homogenate of guinea pig liver, those of 12 to 18 carbons either displayed no effect on oxygen uptake or caused large inhibitions. In his earlier studies Lehninger reported that rat liver homogenates, fortified with adenosine triphosphate and other cofactors, oxidized fatty acids up to 18 carbons, the product being acetoacetate (7, 8). More recently, Kennedy and Lehninger (9) have clarified these earlier results and have reported that the longer chain acids are oxidized essentially to completion, *i.e.* to CO_2 and water, by fortified, washed rat liver particles. In agreement with this finding, O'Connell and Stotz (10) have recently reported that phospholipides of natural origin, when added to washed liver homogenates, increased oxygen consumption. Lehninger (11) also reported that homogenates of rat heart muscle oxidized the higher fatty acids when fortified with adenine nucleotides and fumarate. On the other hand, Grafflin and Green (3) in their studies of the cyclophorase system of rabbit kidney reported results essentially in agreement with those of Muñoz and Leloir (6); *viz.*, that oxygen uptake of the tissue preparations was not increased by acids having more than 12 carbon atoms.

It seems that conclusions depending mainly on differences in oxygen uptake from endogenous levels are subject to several uncertainties. Because of the pronounced inhibitory action of the longer chain acids on the respiration of liver tissue, noted both by Muñoz and Leloir (6) and Lehninger (7, 11), it is conceivable that these acids might be oxidized without displaying a net increase in oxygen consumption. It is also possible that a long chain acid might increase the oxygen consumption of liver preparations without undergoing catabolism, since the fatty acid dehydrogenase responsible for desaturation of the higher acids (*e.g.* stearic to oleic) is particularly abundant in liver (12), and it is conceivable that some portion of the oxygen uptake of liver tissue is utilized for this reaction.

These uncertainties were avoided in the present study by use of the isotopic tracer technique. By using as substrate isotopically tagged palmitic acid (labeled in the carboxyl carbon with C^{13}), its oxidation to CO_2 and its conversion to acetoacetate could be followed simply by measurement of the isotope content of these products. It was thus possible to measure the oxidation of this natural fatty acid in slices and homogenates of rat liver and other organs whose endogenous respiration is ordinarily so high as to overshadow the effects of added substrates. It was also possible, in washed homogenates, to evaluate directly the effect of fumarate on palmitate oxidation without the complicating effect introduced by the oxidation of fumarate itself.

Methods

Preparation of Isotopic Palmitic Acid—The carboxyl-labeled fatty acid was synthesized by the action of C^{13}-tagged CO_2 on the Grignard reagent from pentadecyl bromide. The bromide was prepared in high yield and purity by degradation of silver palmitate, essentially according to the method of Hunsdiecker and Hunsdiecker (13). Two batches of labeled palmitic acid were prepared; the first had an over-all C^{13} excess of 0.84 atom per cent, and the second, which was used in the majority of experiments, had an over-all C^{13} excess of 2.41 atom per cent, corresponding to an excess of 38.6 atom per cent in the carboxyl carbon.

The isotopic acid was prepared for use in these experiments by dissolving it in the calculated quantity of KOH solution to make an 0.01 M stock solution.

Experimental Procedure—Except when effects of feeding and fasting were compared, the animals used were male rats 3 to 6 months of age which had been fasted for 24 hours. Experiments with slices were carried out exactly as described previously (14), except that calcium and magnesium were omitted from the medium to prevent precipitation of palmitate as insoluble soaps. Homogenates were made by the Potter-Elvehjem

technique. The whole homogenates were used directly in 1:5 dilution with isotonic KCl buffered with a 0.01 M phosphate solution, pH 7.6. Washed homogenates were prepared essentially according to procedures of Lehninger and Kennedy (4). In all experiments with homogenates, adenosine triphosphate (Rohm and Haas or Schwarz Laboratories) was added in 0.001 M final concentration. The methods of analysis and calculations were as described previously (14).

Results

Experiments on the oxidation of labeled palmitic acid by various preparations of liver tissue are recorded in Table I. In all experiments with

TABLE I

Oxidation of C^{13}-Labeled Palmitate by Rat Liver Preparations

Approximately 1 gm., dry weight, of tissue (or the equivalent of washed homogenate) was shaken at 37° in 30 ml. of medium. Slices, 2 hours in O_2; whole homogenate, 2 hours in air; and washed homogenate, till oxygen uptake ceased (35 to 40 minutes) in air. Substrate concentration, slices, 0.001 M, homogenates, 0.0005 M. All values were calculated on the basis of 1 gm. or equivalent dry weight of tissue. C^{13} excesses are relative values based on an assumed 100 per cent excess in the palmitic acid.

Preparation	Condition of animal	O_2 uptake	Respiratory CO_2		Acetoacetate	
			Quantity	Relative C^{13} excess	Quantity	Relative C^{13} excess
(1)	(2)	(3)	(4)	(5)	(6)	(7)
		μM	μM	*per cent*	μM	*per cent*
Slices..................	Fasted	733	530	7	52	9
"	"	891	614	5	98	11
"	"	650	343	6	57	11
"	Fed	845	691	5	57	9
Whole homogenate......	Fasted	497	305	8		
" "	"	470	284	7	40	47
" "	Fed	710	418	8	32	32
" "	"	670	460	6	41	27
Washed "	Fasted	84	59	32	16	66
" "	"	98	68	27	17	54

rat liver slices and whole homogenates there appeared in the respiratory CO_2 relative C^{13} excesses of 5 to 8 per cent. Though rather low, these values are significant, since they range from 5 to 20 times the experimental error of the mass spectrometric measurements. In whole homogenates the relative excesses of C^{13} in the respiratory CO_2 were of the same order as in slices; in washed homogenates, the excesses were much higher, reflecting the lower concentrations of endogenous metabolites.

Making the reasonable assumption that all 16 carbon atoms of palmi-

tate are metabolized in the same manner as is the labeled carboxyl carbon, we find that in slices and whole homogenates only 5 to 8 per cent of the total respiratory CO_2 was derived from the labeled acid. This does not indicate, of course, that fatty acid oxidation accounts for only 5 to 8 per cent of the liver respiration. Before reaching the fatty acid oxidases, the labeled acid must come into admixture with the unlabeled acids of the tissue. Without knowing the size of this endogenous fatty acid pool one cannot determine what proportion of the respiration is represented by fatty acid oxidation; however, there is no reason to assume that it does not represent a major share.

The results of these experiments further indicate that, like the short chain acids (14), palmitate is also converted to ketone bodies in the normal "fed" or "fasted" liver (Table I, Columns 6 and 7). Indeed, with homogenates the relative C^{13} excess in the acetoacetate was invariably much higher than in the respiratory CO_2. This result indicates that the carbon of palmitate in homogenates is highly accessible for acetoacetate formation.

Comparison of Palmitate and Octanoate Oxidation in Rat Liver Homogenates—In a previous study (14) it was found that, although rat liver slices oxidized the short chain acids both to CO_2 and to acetoacetate, conversion to acetoacetate accounted for a major share of the fatty acid carbon undergoing catabolism. In contrast, it appeared from these experiments that oxidation of palmitic acid was directed more toward complete oxidation to CO_2. To obtain more decisive information on this point a comparison was made of octanoate and palmitate oxidation by aliquots of the same tissue. The results are given in Table II. To keep the concentration of fatty acid carbon the same for octanoate and palmitate, the respective molar concentrations of the two substrates were 0.001 and 0.0005.

Although in the whole homogenate both substrates yielded approximately equal total quantities of CO_2 and acetoacetate, differences were observed in the respective conversions of each substrate. The isotope data show that about $1\frac{1}{2}$ times as much octanoate carbon was converted to acetoacetate as was completely oxidized to CO_2, whereas somewhat less palmitate carbon was converted to acetoacetate than was oxidized to CO_2.

In the washed homogenate the differences between octanoate and palmitate in their relative conversions to CO_2 and to acetoacetate are far more striking. Here almost 10 times as much octanoate carbon was converted to acetoacetate as was completely oxidized to CO_2, whereas only about $1\frac{1}{2}$ times as much palmitate was converted to acetoacetate as to CO_2. Looked at in another way, twice as much palmitate carbon as octanoate carbon

was completely oxidized, whereas over 3 times as much octanoate carbon as palmitate carbon was converted to acetoacetate. Evidently the shorter chain acids have a greater tendency than the natural long chain acids to be converted to ketone bodies, a finding essentially in agreement with that of Kennedy and Lehninger (9), made on the basis of acetoacetate and respiratory quotient measurements.

Distribution of C^{13} between Carboxyl and Carbonyl Carbons of Acetoacetate—Inasmuch as previous studies showed that an essentially equal distribution of C^{13} between the carboxyl and carbonyl carbons of acetoacetate resulted when carboxyl-labeled octanoate was oxidized by liver slices,

<div align="center">

TABLE II

Palmitate and Octanoate Oxidation Compared in Rat Liver Homogenates

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Conditions same as in Table I. Concentration of substrate, octanoate, 0.001 M; palmitate, 0.0005 M.

Acid	Preparation	Respiratory CO_2			Acetoacetate		
		Total	Relative C^{13} excess	From fatty acid	Total	Relative C^{13} excess	From fatty acid
		μM	per cent	μM	μM	per cent	μM
Octanoate.......	Whole homogenate	473	11	52*	43	47	20†
Palmitate.......	" "	460	8	37	41	18	8
Octanoate......	Washed "	69	18	14	53	64	34
Palmitate.......	" "	92	29	27	22	48	11

* These 52 μM of octanoate carbon represent the complete oxidation of 52/8 = 6.5 μM of octanoic acid.

† These 20 μM of acetoacetate represent 20 × 4 = 80 microatoms of octanoate carbon or 80/8 = 10 μM of octanoic acid.

it was of interest to test the distribution in acetoacetate derived from the labeled palmitate. Such data have been obtained in five experiments and are listed in Table III. The distribution is seen to be equal within the experimental error of the measurements. The data are not sufficiently precise to demonstrate an inequality of distribution such as would be expected if the last 2 carbon atoms of the chain are different from the rest, as was found for octanoate by Crandall, Brady, and Gurin (15). They are, however, in accord with the idea that acetoacetate arises in homogenates as well as in slices, and from longer as well as shorter chain acids by essentially random condensation of acetyl groups split from the fatty acid chain by β oxidation.

Effect of Fumarate on Palmitate Oxidation by Washed Liver Homogenates—In view of the now well established participation of the citric

acid cycle in fatty acid oxidation it was of interest to test the effect of components of the cycle on palmitate oxidation in liver. In preliminary experiments it was found that the addition of fumarate in concentrations up to 0.01 M, though greatly increasing the respiration of slices and crude homogenates, had no marked effect on the incorporation of C^{13} in the respiratory CO_2 during oxidation of labeled palmitate. Presumably components of the cycle are present in these preparations in concentrations sufficient for maximal fatty acid oxidation, and the addition of further

TABLE III

Distribution of C^{13} in Acetoacetate Formed from Carboxyl-Labeled Palmitate

The values are in atom per cent excess.

Preparation	Carbonyl	Carboxyl	$\frac{Carboxyl}{Carbonyl}$
Whole homogenate....................	0.54	0.40	0.7
" "	0.78	0.91	1.2
Washed "	0.30	0.42	1.4
" "	0.51	0.45	0.9
" "	0.27	0.34	1.2
Mean.................................			1.1 ± 0.2

TABLE IV

Effect of Fumarate on Oxidation of Palmitic Acid in Washed Rat Liver Homogenate

Conditions same as in Table I.

Concentration of added fumarate	O₂ uptake	Respiratory CO₂			Acetoacetate		
		Total	Relative C^{13} excess	From fatty acid	Total	Relative C^{13} excess	From fatty acid
M	μM	μM	per cent	μM	μM	per cent	μM
0	120	75	35	26	22	57	13
0.0001	150	91	41	37	17	47	8
0.0005	342	227	33	75	12		

quantities of fumarate has no effect except to increase the respiratory rate by virtue of its own oxidation.

The effect of fumarate on palmitate oxidation in the washed liver homogenate is illustrated in Table IV. Separate aliquots of the same liver preparation in the presence of 0.0005 M palmitate were shaken in large Warburg vessels with varied fumarate concentrations. The addition of fumarate in 0.0001 M final concentration resulted in a definite increase in the incorporation of excess C^{13} in the respiratory CO_2; in 0.0005 M concentration the incorporation was almost 3 times that of the homogenate with no added fumarate.

Effect of Fumarate on Acetoacetate Formation—With increase in fumarate concentration there was a progressive decrease in acetoacetate formation. Table IV shows that with no added fumarate the quantity of palmitate carbon converted to acetoacetate was about $1\frac{1}{2}$ times that converted to CO_2. With 1×10^{-4} M fumarate this ratio became reversed, and although the quantity of acetoacetate formed in the presence of 5×10^{-4} M fumarate was too small for an isotope determination, it is obvious

TABLE V

Oxidation of C^{13}-Labeled Palmitate by Extrahepatic Tissues

Approximately 1 gm. of tissue, dry weight, was shaken in 30 ml. of medium. Muscle tissue was homogenized in a Waring blendor; other tissues by the Potter-Elvehjem method. All experiments were run 2 hours at 38° in air with homogenates and in oxygen with slices. The values are calculated for 1 gm., dry weight, of tissue.

Tissue	Respiratory CO_2	
	Quantity	Relative C^{13} excess
	μM	*per cent*
Pigeon breast muscle...................	273	3.7
" heart...................	354	6.0
Rat skeletal muscle...................	159	0.0
" heart...................	250	2.9
" " slices...................	443	4.1
" brain...................	271	0.0
" "	270	2.1
" kidney...................	375	4.2
" "	258	4.3
" " slices...................	1062	7.5
" " "	812	10.4

that the quantity of palmitate converted to acetoacetate would be relatively still lower.

Oxidation of Palmitic Acid in Extrahepatic Tissues—In Table V there are recorded some preliminary results of a study of palmitate oxidation in extrahepatic tissues. Except for rat skeletal muscle and brain, oxidation of the labeled fatty acid occurred in all of the tissues tested. Though the rate of palmitate oxidation appeared to be higher in pigeon heart and rat kidney slices, these results are considered only of qualitative significance, since it is not known whether the conditions used in these experiments were optimal for palmitate oxidation.

DISCUSSION

Relative Conversion of Palmitate to CO_2 and Acetoacetate—Without definite evidence it has been generally stated that the primary function of the liver in the catabolism of fatty acids was to convert them to ketone bodies;

the further oxidation of these intermediates was presumed to represent an extrahepatic function ((5) p. 408, (16, 17)). This concept arose from various studies *in vitro*, the results of which showed that essentially all of the disappearance of short chain acids in liver slices could be accounted for as increases in ketone bodies (2, 18). Confirmation for this idea was provided by Stadie, Zapp, and Lukens (19) who found that the decrease in endogenous fatty acid content of diabetic cat liver slices after incubation *in vitro* could be accounted for rather completely on the assumption of a conversion of the entire fatty acid molecule to ketone bodies. The fact that acetoacetate is the apparent end-product of the metabolism of short chain fatty acids (and even pyruvic acid) in washed rat liver homogenates (4) can be interpreted also as indicating that the catabolism of fatty acids in liver ends at the ketone body stage. If the data obtained in the present study with palmitic acid can be considered typical of the behavior of the natural fatty acids it must be concluded that quantitatively their complete oxidation to CO_2 in liver is of equal or greater significance than their conversion to acetoacetate, though both reactions occur just as they do with the shorter chain acids.

It is well recognized, however, that processes taking place in a washed liver homogenate may not accurately reflect occurrences in the intact liver cells. It has already been observed (14) that in the relatively intact cells of the liver slice fatty acids of up to 8 carbons are completely oxidized as well as converted to ketone bodies; quantitatively, however, conversion to ketone bodies preponderated greatly over conversion to CO_2.

It might be presumed that the fatty acid is completely oxidized *after* conversion to acetoacetate; *i.e.*, that the complete oxidation of fatty acids involves a "ketolytic" process. Unpublished experiments with isotopic acetoacetate have shown, however, that little if any oxidation of acetoacetate takes place in the rat liver slice.

Effect of Fumarate—During recent years a decisive accumulation of indirect evidence has implicated the citric acid cycle in the oxidation of fatty acids. This concept has been strengthened in the present study with the direct demonstration of an increased rate of oxidation of a natural fatty acid by a component of the citric acid cycle. The effect of fumarate and other citric acid cycle components on the oxidation of long chain fatty acids, particularly in extrahepatic tissues, is still under study. Inasmuch as similar effects have been observed by Lehninger and Kennedy (4), Grafflin and Green (3), and Muñoz and Leloir (6) for the short chain acids, it seems quite evident that the same system of enzymes is involved in the oxidation of both the longer chain and shorter chain acids. Differences in response of oxygen uptake to the addition of the longer chain acids observed by different investigators can probably be attributed to

variations in the amounts of endogenous substrates, to the presence of variable concentrations of activators such as citric acid cycle components, or to variable inhibitions induced by the fatty acids themselves.

Oxidation of Fatty Acids by Extrahepatic Tissues—Despite the lack of direct evidence for the utilization of the higher fatty acids by extrahepatic tissues, a convincing array of indirect evidence for this possibility has been cited by Stadie ((5) p. 417), and more recently further indications of direct fat oxidation by peripheral tissues have been advanced in studies of the oxidation of the shorter chain acids by tissue preparations *in vitro* (3, 20). The present study substantiates and extends the proof for the occurrence of extrahepatic oxidation of natural fatty acids.

SUMMARY

A study of the oxidation of isotopic palmitic acid by various animal tissues *in vitro* revealed that in rat liver this acid can be converted both to carbon dioxide and to acetoacetate. In contrast with the lower fatty acids (up to C_8) which are transformed preponderantly to ketone bodies, palmitate is converted about equally to carbon dioxide and to acetoacetate by slices and homogenates of rat liver.

In washed homogenates of rat liver, fumarate in low concentrations markedly accelerates oxidation of palmitate to CO_2 and at the same time decreases the formation of ketone bodies. This finding directly confirms previous evidence implicating the citric acid cycle in the oxidation of the natural fatty acids.

Palmitic acid was found to be oxidized *in vitro* by a variety of tissues besides liver.

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A QUANTITATIVE COLOR REACTION FOR CORTISONE AND RELATED 17,21-DIHYDROXY-20-KETOSTEROIDS

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The numerous combinations of functional groups occurring in the steroid hormones render difficult the development of a specific test for individual compounds. However, reactions which reveal certain groupings are available and may be applied singly or in combination to elucidate the characteristics of pure steroids or of compounds in a mixture.

The ketol side chain, located at carbon atom 17 in certain corticosteroids, has reducing properties and also yields formaldehyde upon oxidation with periodic acid. These reactions have been applied in the analysis of partially purified steroid mixtures (1–4). The reaction of active carbonyl groups in steroids with phenylhydrazines is the basis of histochemical methods (5). Production of colored and fluorescent solutions in sulfuric acid (6) and reaction with antimony trichloride (7), dinitrobenzene (8), and other reagents (9) have also been employed.

To this group may now be added another test which appears to be specific for 17,21-dihydroxy-20-ketosteroids. The method is based upon the appearance of a yellow color in solutions containing the steroid, phenylhydrazine, and sulfuric acid.

EXPERIMENTAL

Color Reaction

The effects of changes in heat treatment and concentration of phenylhydrazine and sulfuric acid upon the production of color with cortisone acetate are demonstrated in Tables I and II. Phenylhydrazine in sulfuric acid absorbs light at 410 mμ, and certain corticosteroids develop colors in sulfuric acid more concentrated than about 1.8:1. For these reasons, use of phenylhydrazine and sulfuric acid in concentrations somewhat less than optimal is recommended. The following procedure has been found to be satisfactory.

Reagents—

Dilute sulfuric acid (1.63:1). 190 ml. of water plus 310 ml. of concentrated acid.

Phenylhydrazine hydrochloride. Recrystallized from ethanol and dried over CaCl$_2$.

TABLE I

Influence of Temperature upon Rate of Reaction of Cortisone Acetate with Phenylhydrazine and Sulfuric Acid

Time	Optical density* at 410 mμ				
	50 γ cortisone acetate in 1 ml. methanol and 2 mg. phenylhydrazine HCl in 8 ml. 1.27:1 sulfuric acid and water				
	50°	60°	70°	80°	100°
min.					
2		0.014	0.023	0.082	0.198
4		0.046	0.087	0.193	0.184
8		0.134	0.201	0.228	0.142
16	0.139	0.227	0.249	0.210	0.097
24	0.194	0.254	0.259	0.176	
32	0.233	0.259	0.246	0.151	

50 γ cortisone acetate in 1 ml. methanol and 5 mg. phenylhydrazine HCl in 8 ml. 1.63:1 sulfuric acid and water, temperature, 60°

	4 min.	8 min.	16 min.	24 min.	32 min.
	0.093	0.216	0.287	0.294	0.290

* $-$Log T.

TABLE II

Influence of Reagent Concentrations upon Color Reactions

Phenylhydrazine	Optical density at 410 mμ	Average increase in optical density per mg. phenylhydrazine HCl
50 γ cortisone acetate in 1 ml. methanol and phenylhydrazine HCl in 8 ml. 1.27:1 sulfuric acid and water. 10 min. at 60°		
mg.		
1	0.124	0.046
2	0.170	0.018
4	0.206	0.006
8	0.229	0.0016
16	0.242	
50 γ cortisone acetate in 1 ml. methanol and 2 mg. phenylhydrazine HCl in 8 ml. of sulfuric acid and water. 10 min. at 60°		
Sulfuric acid-water, by volume		
0.67:1	0.018	
0.92:1	0.080	
1.27:1	0.177	
1.78:1	0.238	
2.56:1	0.196	

Phenylhydrazine in acid. 65 mg. of the hydrochloride in 100 ml. of dilute sulfuric acid, prepared fresh each day.

Method—To a steroid sample in 1 ml. of methanol are added 8 ml. of phenylhydrazine in acid and the solution is heated at 60° ± 1° for 20 minutes. It is cooled in running tap water for 3 minutes, and its optical density (a) is compared with that of a methanol-sulfuric acid-phenylhydrazine blank at 410 mμ.[1]

In order to correct for interfering substances which yield colored solutions in sulfuric acid, a duplicate sample is treated as above with phenyl hydrazine omitted. The optical density (b) is compared with that of a methanol-sulfuric acid blank at 410 mμ. The difference in optical den-

TABLE III

Relationship between Amount of Cortisone Acetate and Optical Density at 410 mμ

Steroid in 1 ml. of methanol, 5 mg. of phenylhydrazine hydrochloride in 8 ml. of 1.63:1 sulfuric acid and water. 20 minutes at 60°.

Cortisone acetate	Optical density	Optical density per γ
γ		
1	0.0065	0.0065
2	0.0120	0.0060
5	0.0315	0.0063
10	0.0657	0.0066
25	0.157	0.0063
50	0.295	0.0059
100	0.555	0.0056

sities, $a-b$, is proportional to the quantity of 17,21-dihydroxy-20-ketosteroid in the sample.

The residues left upon drying chloroform extracts of blood plasma frequently yield opalescent solutions in methanol-sulfuric acid. A correction for interference due to opalescence may be made by subtracting 1.5 times the optical density at 600 mμ from the optical density at 410 mμ. This correction factor was determined by comparing optical densities of opalescent, colorless solutions at the two wave-lengths. The factor, 1.5, applies only to the instrument and cuvette specified.[1]

Variation of Optical Density with Amount of Steroid—With 1 to 25 γ of pure cortisone acetate, the optical density varied linearly with the amount of steroid in the solution (Table III). Under the conditions, the colorimetric method was sensitive to about 1 γ of cortisone acetate.

Specificity—Of the steroids tested, only cortisone, cortisone acetate, and 17-hydroxy-11-desoxycorticosterone acetate reacted with phenylhy-

[1] The Coleman universal spectrophotometer, model 11, with a 0.5 inch square cuvette, was used.

drazine and sulfuric acid to produce solutions exhibiting an absorption band at 410 mμ (Table IV). Without phenylhydrazine, these steroids did not produce appreciable color in sulfuric acid.

Methyltestosterone, estradiol, pregnenolone, and pregnentriolone yielded colored solutions, with absorption maxima at 460 to 480 mμ, in sulfuric

TABLE IV

Reaction of Steroids with Phenylhydrazine in Sulfuric Acid

50 γ of steroid in 1 ml. of methanol plus 8 ml. of 1.63:1 sulfuric acid and water or 5 mg. of phenylhydrazine HCl in 8 ml. of acid. 20 minutes at 60°.

Steroid*	Optical density at 410 mμ in		Absorption maximum
	H_2SO_4	H_2SO_4 + phenylhydrazine	
			mμ
Cortisone acetate	0.000	0.342	410
Cortisone	0.000	0.362	410
17-Hydroxy-11-desoxycorticosterone acetate	0.007	0.171	410
11-Desoxycorticosterone acetate	0.015	0.011	
Corticosterone	0.015	0.018	
11-Dehydrocorticosterone acetate	0.000	0.011	
Δ⁴-Androstene-3,17-dione	0.000	0.007	
Δ⁴-Androstene-3,11,17-trione	0.004	0.013	
Methyltestosterone	0.063	0.036	475
Estradiol	0.074	0.029	460
Δ⁵-3-Hydroxypregnenone-20	0.065	0.039	480
Δ⁴-Pregnene-17,20,21-triol-3-one	0.097	0.108	465
Testosterone propionate	0.004	0.000	
17-Hydroxyprogesterone	0.007	0.007	

* The methyltestosterone, testosterone propionate, and estradiol were purchased from the Delta Chemical Works, New York. The 17(α)-hydroxyprogesterone was generously supplied by Dr. P. L. Julian, The Glidden Company, Chicago, and the remaining steroids by Dr. L. H. Sarett and Dr. M. Tishler, Research Laboratories, Merck and Company, Inc., Rahway.

acid. At 410 mμ these solutions, except the one containing pregnentriolone, absorbed more light than solutions which contained phenylhydrazine also. It is obvious that if large quantities of these steroids are present in a mixture an erroneously high blank value may be encountered. However, precise estimation of cortisone acetate was possible in a solution containing 20 γ each of this steroid, methyltestosterone, estradiol, and pregnenolone. A number of substances such as fructose and dehydroascorbic acid produce color with the reagent, but in the preparation of the sample, these compounds, as well as lipide-soluble, volatile materials, are eliminated if the sample is extracted with chloroform and the extract dried.

Phenylhydrazine Derivatives—α-Methylphenylhydrazine reacted with the 17,21-dihydroxy-20-ketosteroids in acid similarly to phenylhydrazine. 2,4-Dinitrophenylhydrazine was inferior in the production of color and its yellow color interfered with the spectrophotometric determinations.

Resorcinol also gave a yellow color with cortisone in sulfuric acid. This reaction was not further investigated because the absorption peak at 465 mμ overlapped the peak obtained with some steroids in sulfuric acid alone.

Application

Adrenal Cortex Extract[2]—2 ml. of the aqueous solution, which on direct assay was found to contain 150 γ of 17,21-dihydroxy-20-ketosteroids, calculated as cortisone, were extracted with 5 ml. of chloroform. The chloroform extract contained all of the chromogenic material. An aliquot of the extract was dried in a stream of nitrogen at 40°, and the residue was dissolved in 0.5 ml. of methanol plus 7 ml. of benzene. This solution was extracted four times with 10 ml. of water. Each aqueous solution

TABLE V

Benzene-Water Partition of Cortisone and of 17,21-Dihydroxy-20-ketosteroids from Adrenal Extract

Fraction	Per cent in fraction	
	Cortisone	Adrenal steroids
Aqueous Extract 1	40.2	59.7
" " 2	21.3	21.5
" " 3	13.4	7.4
" " 4	8.2	4.6
Benzene residue	13.0	6.8

was extracted with 10 ml. of chloroform, and each chloroform extract was divided into two equal portions, dried, and analyzed by the color reaction. These residues yielded colors in sulfuric acid roughly half as intense as in the phenylhydrazine reagent. The data from this experiment are summarized in Table V. For comparison, data obtained in a parallel experiment with pure cortisone are recorded.

Lipoadrenal Cortex[3]—0.2 ml. of the oil solution was dissolved in 0.3 ml. of methanol and 7 ml. of benzene. Four 10 ml. aqueous extracts of the

[2] Upjohn (licensed under Rogoff and Stewart, U. S. patent 2,096,342, 1937). Each ml. was obtained from not less than 40 gm. of adrenal glands and contains not less than 50 dog units (2.5 rat units) when assayed by the method of Cartland and Kuizenga (10).

[3] Upjohn. Each ml. contains 40 rat units (survival-growth) and is standardized by the work-performance test to be equivalent in biological activity to 2 mg. of 17-hydroxycorticosterone.

benzene solution contained 24.5, 7.4, 4.5, and 2.0 γ of 17,21-dihydroxy-20-ketosteroids, calculated as cortisone.

The acetate of neither cortisone nor 17-hydroxy-11-desoxycorticosterone is readily extractable from benzene with water. From the data of Talbot *et al.* (1), it is reasonable to assume that free 17-hydroxy-11-desoxycorticosterone would be less readily extracted than cortisone. In view of these considerations, and because the chromogenic material in both the aqueous and lipo extracts was more easily extracted from benzene-methanol with water than was cortisone, it is possible that the adrenal extracts contained water-soluble derivatives of cortisone or closely related steroids.

Animal Blood and Urine—A 10 kilo dog was given a subcutaneous in-

TABLE VI

Determination of Cortisone Added to Blood Plasma and Urine

10 ml. of plasma or urine plus cortisone in 1 ml. of methanol. The plasma was extracted with 40 ml. of chloroform; 15 ml. of extract used for assay. The urine was extracted with 10 ml. of chloroform; 4 ml. of extract used for assay.

Cortisone added	Cortisone found		Recovery	
	Plasma	Urine	Plasma	Urine
γ *per ml.*	γ *per ml.*	γ *per ml.*	*per cent*	*per cent*
0	0.0	0.21		
1.0	0.95	1.05	95	85
1.5	1.23	1.56	82	90
2.0	1.63	1.97	82	88
2.5	2.24	2.48	90	91
Mean ± s.e....................			87 ± 3.2	89 ± 1.5

jection of 100 mg. of cortisone acetate in saline suspension. Blood was drawn from the jugular vein 1, 2, 6, and 10 hours after the injection, and urine was collected for 2 days. Cortisone, added to blood plasma or urine, can be transferred to chloroform by a single extraction, and, after evaporation of the chloroform, determined by the colorimetric method with an accuracy of 85 to 90 per cent (Table VI) and a precision of 2 to 4 per cent (standard error of the mean). However, no trace of 17,21-dihydroxy-20-ketosteroids was found in either the plasma or the urine of this dog. No evidence for the excretion of conjugated cortisone was revealed by acid hydrolysis of the urine (1 N H_2SO_4 at 100° for 10 to 30 minutes). However, this treatment destroyed nearly 50 per cent of added cortisone (10 γ per ml.). It appears that the steroid was rapidly destroyed in the animal, fixed by the tissues, or converted to a form which was not extractable with chloroform.

Likewise, no cortisone could be detected in the plasma of rats 1, 2, or 4 hours after they had received an intraperitoneal injection of 1 mg. of cortisone acetate per 250 gm. of body weight, administered as a solution in propylene glycol. In another experiment, normal and adrenalectomized rats, three animals per group, were injected subcutaneously daily with 3 mg. of cortisone acetate in saline suspension. On the 5th day of dosing, these animals excreted an average of 22 γ (normal) and 12 γ (adrenalectomized) of chloroform-extractable 17,21-dihydroxy-20-ketosteroids in the urine.

SUMMARY

A color reaction which appears to be specific for 17,21-dihydroxy-20-ketosteroids is described. Adrenal extracts have been found to contain compounds of this class.

No substances responding to the test could be extracted from dog plasma and urine or rat plasma with chloroform following single subcutaneous or intraperitoneal injection of cortisone acetate. After prolonged administration, small quantities of such compounds appeared in the urine of rats.

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MODE OF INHIBITION OF CHYMOTRYPSIN BY DIISOPROPYL FLUOROPHOSPHATE

II. INTRODUCTION OF ISOPROPYL AND ELIMINATION OF FLUORINE AS HYDROGEN FLUORIDE*

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Considerable practical importance is attached to the mode of inhibition of chymotrypsin by diisopropyl fluorophosphate (DFP), because it is evident that this inhibition reaction, fairly general among esterases, may furnish a clue to the nature of the active grouping in this enzyme, and may also explain the mechanism of the toxicity of DFP together with a number of related substances whose industrial applications are becoming fairly wide-spread, and therefore a matter of some public concern.

Trypsin and chymotrypsin are both inhibited by small amounts of DFP (1). The esterolytic and proteolytic activity of each enzyme is inhibited at the same rate and to the same extent, thus indicating the probability that the two activities reside in the same "active centers." Approximately 1 mole of inhibitor is required for the complete inhibition of chymotrypsin, whereas trypsin requires more inhibitor. The reaction time is very brief. An enzymically inert, crystalline protein has been obtained as a reaction product of α-chymotrypsin with slightly more than 1 mole of DFP per mole of enzyme. By the use of DFP containing radioactive phosphorus, it was found that phosphorus had been introduced into the crystalline, inhibited protein to the extent of 1.1 moles per mole of enzyme (2), a value in excellent agreement with that calculated from the amount needed for 50 per cent inhibition. These equivalents are calculated with 27,000 as the molecular weight of α-chymotrypsin, determined by light-scattering measurements[1] in this Laboratory on carefully purified samples of the enzyme. The molecular weight of the inhibited protein as determined by osmotic pressure and the light-scattering method was found to be substantially the same as that of the original enzyme.

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* Enzyme Research Division Contribution No. 127.

[1] These results, the details of which will be published later, were obtained in conjunction with Dr. K. J. Palmer of the Western Regional Research Laboratory.

Analysis of the crystalline, inhibited protein by the Zeisel procedure showed that (approximately) two isopropyl groups were introduced into chymotrypsin by treatment with DFP. Fluorine, however, was found to be absent on spectroscopic analysis. Furthermore, approximately 1 mole of acid (apparently HF) was formed during the inhibition reaction; thus it is apparent that DFP reacts with α-chymotrypsin through a condensation reaction with the formation of HF.

Attempts were made to determine which amino acid residue of chymotrypsin reacted with DFP. The amino nitrogen of the inhibited protein was identical with that of chymotrypsin. Furthermore, the electrophoretic mobility of the former was identical with that of the latter. This may suggest that neither an amino nor a carboxyl group is involved in the inhibition reaction, but it is not at all conclusive evidence, since the reaction of only one such group per molecule may not be detectable by these means.

The effect of twenty amino acids on the inhibition reaction was determined. In no case did an amino acid interfere with the inhibition. Furthermore, an acid hydrolysate of chymotrypsinogen also failed to interfere. Thus the group which reacts with DFP appears to owe its reactivity to factors involving the configuration of something larger than the individual amino acids.

It may be of interest to note that rather good preliminary evidence was obtained to the effect that a terminal threonine group in chymotrypsin combines with DFP. Periodate oxidation was found to liberate approximately 1 mole of acetaldehyde from each mole of chymotrypsin, whereas practically no acetaldehyde was recovered from the crystallized, inhibited protein. However, this was later shown to be due to a small amount of impurity in the chymotrypsin. The active enzyme could be oxidized with periodate to give the maximum amount of acetaldehyde without interfering with the activity as measured on several substrates. In addition, no terminal threonine disappeared during the inhibition reaction when the entire reaction mixture (rather than the crystalline end-product) was considered. Furthermore, after the inhibition it was possible to isolate a part of the impurity which contained terminal threonine to the extent of 1 mole of threonine per 4000 gm. Hence efforts are being made to isolate the residue to which the diisopropyl phosphate group is attached.

Materials and Methods

The chymotrypsin used in this work was α-chymotrypsin, either purchased[2] or prepared from chymotrypsinogen. The methods of assay were

those used previously (1), except that hydrolysis of the tyrosine ethyl ester (TEE) was carried out at pH 6.25.[3] The milk clotting procedure was that of Kunitz (8). Threonine determinations were done on the intact protein according to the method of Shinn and Nicolet (9) with the use of 0.004 N iodine to titrate the bisulfite which was bound. Clark's modification (10) of the Viebock and Schwappach method for the determination of alkoxy groups was used with $CdCl_2$ in the trap. The digestion was run for 30 minutes (the time for the usual Zeisel determination) and also for 6 hours (the time for the Baernstein methionine determination (11)). The difference between the volatile alkyl iodide obtained from α-chymotrypsin and from its inhibited product was the same regardless of digestion time, although, of course, the total amount was greater in both cases after the longer digestion.

Results

Isopropyl Groups in DFP-Inhibited α-Chymotrypsin—Twice crystallized, dialyzed, and lyophilized DFP-inhibited α-chymotrypsin, whose preparation was described previously (1), was used for the determination of isopropyl groups. Since α-chymotrypsin contains methionine (3), which gives CH_3I slowly on refluxing with HI, it was necessary to determine the isopropyl groups by the difference between the uninhibited and inhibited protein when the reaction mixtures were digested for the same length of time. Digestion periods of 0.5 hour (the time necessary for quantitative conversion and distillation of the alkyl groups on oxygen) showed that treatment with DFP had introduced 2.2 moles of isopropyl per mole of enzyme (Table I). Since a 0.5 hour digestion is known not to give quantitative conversion of methionine to CH_3I (11), it might be reasoned that part of the difference noted was due to a difference in rate of formation of CH_3I in the inhibited and uninhibited enzyme. Hence the recommended digestion time of 6 hours for methionine was likewise used. This showed the introduction of 1.7 moles of isopropyl groups per mole of inhibited protein (Table I), a result in substantial agreement with that from the short digestion period when the small isopropyl content is considered. Hence it is apparent that the isopropyl groups, as well as the phosphorus, are introduced into chymotrypsin through the inhibition reaction.

[3] A. L. Curl of our laboratory has found that the pH optimum for the hydrolysis of TEE by chymotrypsin was at pH 6.25, whereas that for hemoglobin is at pH 7.8 (3). This, together with the effect of methanol (4, 5), may account for the failure of Kaufman and Neurath (6) to observe much hydrolysis of TEE, since at pH 7.8, at which their assay was run, the rate of splitting is only about one-seventh of the maximum rate. This difference in optimum pH between the ester and protein hydrolysis is contrary to what has been observed for trypsin (7), and emphasizes the importance of determining the pH optimum for each new substrate.

Formation of HF during Inhibition Reaction and Lack of Fluorine in DFP-Inhibited α-Chymotrypsin—Acid was observed to form during the reaction of α-chymotrypsin with DFP in an amount equivalent to the amount of enzyme protein used. This was shown by the following experiments. To a solution of 1.42 gm. (0.0526 mM) of α-chymotrypsin in 84 ml. of 0.01 M phosphate buffer at pH 7.7, 0.84 ml. of 0.1 M DFP (0.084 mM) in isopropanol was added. The acid produced was titrated electrometrically with 0.02 N NaOH. The production of acid was essentially complete within 2 minutes after the introduction of the DFP[4] and amounted

TABLE I

Isopropyl Groups in DFP-Inhibited α-Chymotrypsin (α-Xt)

Material	Sample*	Digestion time	M.eq. alkyl iodide		Moles isopropyl per mole of DFP-α-Xt†
			Per sample × 10⁴	Per gm.	
	mg.	*hrs.*			
α-Xt	45.8	0.5	5.9	0.013	
"	39.9	0.5	5.1	0.013	
DFP-α-Xt........	30.3	0.5	22	0.077	2.1
"	37.7	0.5	34	0.084	2.3
α-Xt..............	59.2	6.0	48	0.081	
"	65.4	6.0	53	0.081	
DFP-α-Xt........	51.9	6.0	77	'0.149	1.8
"	51.6	6.0	73	0.141	1.6
Average........					1.95

* Corrected for moisture content.

† This is the excess of alkyl iodide obtained from the DFP-inhibited α-chymotrypsin over that from normal α-chymotrypsin, calculated as isopropyl, on the basis of 27,000 for the molecular weight·of both normal and inhibited α-chymotrypsin.

to 3.16 ml. of 0.02 N NaOH, or 0.0632 m.eq., after correction for the acidity of the DFP solution added (owing to the slow decomposition of DFP in isopropanol solutions). In a duplicate experiment 0.057 m.eq. of acid was produced. Thus, 1.2 and 1.1 equivalents of acid were formed per mole of enzyme during the reaction. In view of the inaccuracies involved in such small measurements, it is evident that for each mole of enzyme inhibited 1 mole of acid is produced. The formation of the acidity cannot be due to the hydrolysis of DFP to HF and diisopropyl phosphate,[5]

[4] It was previously shown that the inhibition of chymotrypsin by DFP is extremely rapid (1).

[5] Diethyl phosphate has a pK value of 1.39 (12).

since both of these products are strong acids and 2 moles of acid would result. It might be reasoned that 1 mole of acid disappeared to form the inhibited enzyme after this hydrolysis. However, since neither fluoride ion (13) nor diisopropyl phosphate[6] is inhibitory, such a hypothesis seems highly improbable.

The absence of fluorine in the DFP-inhibited α-chymotrypsin was established by E. J. Eastmond of the Western Regional Research Laboratory by spectrographic analysis. Standards were set up with crystalline β-lactoglobulin to which NaF had been added in varying amounts corresponding to 0 to 0.08 per cent fluorine in the protein. These samples were ashed at 500° for 3 hours in the presence of an excess of $Ca(NO_3)_2$ with NaCl as a carrier. The intensity of the CaF_2 emission band at 5291 A was a measure of the fluorine content. When α-chymotrypsin and its DFP inhibition product were subjected to this analysis, considerably less than 0.02 per cent of fluorine was found in both cases. If fluorine had been introduced to the same extent as were the isopropyl and phosphate groups of DFP, the inhibited product should have contained approximately 0.08 per cent F. The absence of fluorine thus serves as additional evidence that HF was formed during the inhibition reaction. This reaction may accordingly be written

$$
\text{Xt---H} + \text{F---P} \begin{matrix} \text{O} \quad \text{OC}_3\text{H}_7 \\ \| \diagup \\ \diagdown \\ \text{OC}_3\text{H}_7 \end{matrix} \longrightarrow \text{Xt---P} \begin{matrix} \text{O} \quad \text{OC}_3\text{H}_7 \\ \| \diagup \\ \diagdown \\ \text{OC}_3\text{H}_7 \end{matrix} + \text{HF}
$$

where H represents some essential active hydrogen and Xt, chymotrypsin.

Amino Nitrogen and Electrophoretic Analysis of DFP-Inhibited α-Chymotrypsin—The amino nitrogen content (14) of the inhibited enzyme was found to be the same as that of control α-chymotrypsin. However, if the introduction of the isopropyl phosphate group were on an amino group, the decrease in amino nitrogen, on the 1:1 basis, would have been only 0.05 per cent.

In an attempt to obtain additional evidence on this point, W. H. Ward of the Western Regional Research Laboratory determined the electrophoretic mobility of the inhibited and the uninhibited enzyme. The results, given in Table II, show that the DFP inhibition caused no change in the mobility of α-chymotrypsin. These results may be interpreted

[6] We have used the chymotrypsin inhibition as a measure of the rate of hydrolysis of DFP in aqueous solutions, since the rate of inhibition is very rapid as compared with the hydrolysis. Aliquots of aqueous DFP were added to solutions of chymotrypsin and the amount of inhibition was a measure of the residual DFP.

as indicating that neither an amino nor a carboxyl group reacted with the DFP. This evidence must be considered with reservations, as the method may not be sufficiently precise to detect the disappearance of only one ionizable group per mole of protein. However, McMeekin *et al.* (15) found that the combination of 2 molecules of dodecyl sulfate with 1 molecule of β-lactoglobulin (molecular weight 35,000) caused an easily detectable change in the electrophoretic mobility of this protein.

Failure of Amino Acids to Interfere with Inhibition by DFP—In order to ascertain whether DFP might react with a particular amino acid, the inhibition of α-chymotrypsin was determined in the presence of the amino acids. The experiments were so arranged that a large excess of amino acid was included in a system containing chymotrypsin, and only enough DFP was added thereto to inhibit approximately half the enzyme. A

TABLE II

Electrophoretic Mobilities of α-Chymotrypsin and DFP-Inhibited α-Chymotrypsin

pH*	Mobility × 10⁵†	
	α-Xt	DFP-α-Xt
3.46	+4.89	+4.91
5.73	+2.46	+2.47

* Acetate buffers of 0.1 ionic strength were used. The protein concentration was 1.0 per cent.

† Mobilities are reported in cm.^{2} volt^{-1} sec.$^{-1}$ referred to water at 0°.

reaction between DFP and an amino acid should therefore markedly decrease the inhibition obtained. However, in no instance did the amino acids interfere with the inhibition reaction (Table III), even though each was used in an amount much greater than that present in chymotrypsin. On the supposition that chymotrypsin might contain an unknown amino acid which reacts with DFP, the inhibition reaction was carried out in the presence of a neutralized acid hydrolysate of chymotrypsinogen. In the presence of an aliquot of the hydrolysate representing 10 times the amount of α-chymotrypsin used, the inhibition reaction proceeded to the same extent as in its absence. Hence if there is an amino acid in chymotrypsin which reacts in the free state with DFP, it appears to be acid-labile. It seems more likely that the reacting group in the protein must be reactive by virtue of some particular configuration in the protein molecule; quite possibly the same configuration makes the protein an enzyme.

Reaction of α-Chymotrypsin with Periodate—Because of the relatively large threonine content (11 per cent) of chymotrypsinogen and the chymotrypsins (3) and since the conversion of the zymogen to the active enzyme

involves hydrolysis, this conversion might conceivably involve the formation of terminal threonine (*i.e.*, threonine in which only the carboxyl group is in peptide linkage). Analysis of intact α-chymotrypsin for terminal threonine by the method of Shinn and Nicolet (formation of acetaldehyde

TABLE III

Failure of Amino Acids to Interfere with DFP Inhibition Reaction

Group No.	Amino acid added	Chymotrypsinogen content*	Amount added to chymotrypsin	Inhibition†
		per cent	*per cent*	*per cent*
	None			65
1	Arginine	2.83	14	
	Glycine	5.3	26	
	Methionine‡	1.22	6	
	Serine‡	11.4	57	59
2	Glutamic acid	9.0	45	
	Aspartic "	11.3	57	
	Isoleucine‡	5.7	28	
	Leucine	10.4	52	50, 70
3	Alanine‡		50	
	Phenylalanine‡	3.6	18	
	Tryptophan	5.57	28	
	Threonine‡	11.4	57	58
4	Valine‡	10.1	51	
	Proline	5.9	30	
	Hydroxyproline	0	50	
	Lysine	8.0	40	60
5	Tyrosine	2.96	15	
	Norleucine		50	
	Asparagine		50	
	Histidine	1.23	6	58
6	Cysteine	0.6	3	
	Cystine	6.51	37	67

* The values for chymotrypsinogen were used since the amino acid composition of α-chymotrypsin is not completely known (3).

† The inhibition reactions were carried out in 0.2 M phosphate at pH 7.7. The amino acids were grouped for convenience. α-Chymotrypsin was dissolved in the buffered amino acid solutions (0.5 mg. per ml.) and sufficient DFP added to cause 65 per cent inhibition in the absence of amino acids. The extent of inhibition was determined by esterase assays.

‡ The DL-amino acids were used.

with periodate) (9) showed such threonine to be present. Indeed the first preparations analyzed contained almost exactly one such group per mole. Furthermore, the terminal threonine content of the crystalline protein after inhibition with DFP was found to be only one-tenth as much

as in the normal enzyme, thus suggesting that a terminal threonine group is the point of attack of DFP. This, however, was found not to be the case. The hypothesis was abandoned because of the following evidence.

(a) When α-chymotrypsin preparations were subjected to several additional recrystallizations, they contained less terminal threonine. Protein recrystallized seven times contained only 0.65 mole of terminal threonine per mole of enzyme, an obvious impossibility if the terminal threonine were an integral part of the protein. However, all samples of chymotrypsin, whether recrystallized from a commercial product or prepared from the zymogen in this Laboratory, were found to give some acetaldehyde on oxidation with periodate. When the analytical difficulties of accurately determining small amounts of acetaldehyde are considered, additional evidence seems necessary before the hypothesis is discarded.

(b) Periodate oxidation did not destroy the activity of chymotrypsin. As shown in Fig. 1, the amount of acetaldehyde formed by the oxidation of five times crystallized α-chymotrypsin with periodate increased with increasing periodate concentration until a maximum was reached when each mole of enzyme was oxidized with 2.5 moles of periodate. Yet at this point none of the activities was impaired. Oxidation with larger amounts of periodate did, however, cause a decrease in the activities, amounting at most to a loss of 65 per cent of the original (at the molar ratio of 5). Still larger amounts of periodate caused practically no further decrease. The effect of relatively large amounts of periodate on chymotrypsin will be discussed below. Since, however, it was possible to oxidize α-chymotrypsin to the extent of the maximum amount of acetaldehyde formation without any loss in activity, it seemed improbable (though not impossible) that the terminal threonine is associated with the active center and was the point of attachment of the diisopropyl phosphate group.

(c) No terminal threonine was lost during the inhibition reaction, provided that the analysis was made on the entire reaction mixture rather than on the purified inhibited protein. When 190 mg. (0.07 mM) of α-chymotrypsin were treated with 0.09 mM of DFP and the whole reaction mixture oxidized after 20 minutes with an excess of periodate (2 mM), 0.0031 mM of acetaldehyde was formed. A like sample of the same chymotrypsin alone gave on oxidation 0.0030 mM of acetaldehyde. Hence no terminal threonine disappeared during the reaction with DFP, though terminal threonine was not found in the isolated end-product of the reaction.

The foregoing results indicate that even carefully purified chymotrypsin contains an impurity which carries a terminal threonine group. Such a substance has now been isolated from α-chymotrypsin. A solution con-

taining 9.75 gm. or 0.36 mM of three times crystallized α-chymotrypsin in 1100 ml. of 0.2 M phosphate buffer of pH 7.7 was treated with 0.8 mM of DFP. This resulted in complete inhibition. The pH of the solution was adjusted to 4.0 with 60 ml. of 10 per cent H_2SO_4 and sufficient analytical grade $(NH_4)_2SO_4$ was added to make the solution 0.8 saturated.
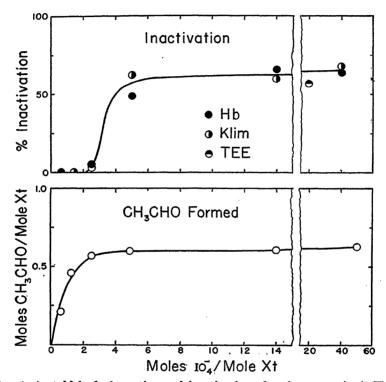

FIG. 1. Acetaldehyde formation and inactivation of α-chymotrypsin (α-Xt) by sodium periodate. The proper amount of sodium periodate was added to a solution of 200 mg. of five times crystallized α-chymotrypsin in 15 ml. of 0.1 M phosphate buffer at pH 7.8. The acetaldehyde was removed by a stream of CO_2 passed through the reaction mixture for 1 hour (9), and the activities were determined immediately thereafter.

The precipitated protein was filtered off and dissolved in 115 ml. of 0.01 N H_2SO_4. A gelatinous, slightly yellow precipitate remained undissolved and was filtered off, washed several times with water, and analyzed. The material (142 mg.) contained 7.4 per cent of nitrogen and 2.9 per cent of terminal threonine. The material thus isolated accounted for 0.035 mM or 20 per cent of the total terminal threonine in the α-chymotrypsin used.

If all of the impurity containing this residue had the same equivalent weight (on the terminal threonine basis) as that isolated, then the α-chymotrypsin would contain approximately 7 per cent of this impurity. However, it is probable that only the higher molecular weight fraction of the impurity was isolated, since this impurity is not likely to be a single substance. The proportion of impurity in the α-chymotrypsin is therefore apt to be considerably lower than the maximum value as calculated from the foregoing data. The great tenacity with which this material accompanies α-chymotrypsin during recrystallization suggests that it may be attached to the enzyme in a manner analogous to a substrate, but incapable of hydrolysis. This idea, coupled with the fact that the material is displaced from the protein by DFP during the reaction of inhibition, leads naturally to the supposition that the place of attachment of the threonine-bearing impurity is also the grouping that combines with DFP.

Tetraethyl Pyrophosphate (TEP) Inhibition of α-Chymotrypsin and Trypsin—TEP was previously reported to be without effect on trypsin when the enzyme was exposed to the inhibitor for 20 minutes previous to assay (1). However, an extension of this work has shown trypsin to be inhibited by TEP but at a very slow rate; e.g., when 2.5 mg. of trypsin dissolved in 100 ml. of 0.1 M phosphate buffer at pH 7.0 were incubated at 5° with sufficient TEP to make the solution 1×10^{-4} M, only 5 per cent inhibition (determined by esterase activity (7) with α-toluenesulfonyl-L-arginine methyl ester as a substrate) was obtained after 1 hour. After 6 and 18 hours, 43 and 63 per cent inhibition, respectively, occurred. Under these conditions of temperature and pH, trypsin itself was found to be very stable. At pH 5.0 and 25°, with the same amounts of trypsin and TEP, only 15 per cent inhibition was observed after 30 hours. Hence TEP inhibits trypsin, but at a very slow rate. These results emphasize again that, in order to define the inhibitory properties of these phosphate esters, the time as well as the concentrations must be considered (13).

In confirmation of the results of Jandorf *et al.* (16), TEP was found to inhibit α-chymotrypsin. When a solution of this enzyme (17 mg. per 10 ml.) in 0.1 M phosphate buffer at pH 7.0 was incubated with sufficient TEP to make the reaction mixture 1×10^{-4} M, 50, 80, and 90 per cent inhibition was found after 0.3, 1, and 18 hours, respectively. This rate of inhibition was considerably more rapid than that observed with trypsin but much slower than the rate of inhibition of chymotrypsin by DFP (1).

DISCUSSION

The fact that the inhibition of α-chymotrypsin by DFP was not interfered with by any of the amino acids used or by a hydrolysate of chymo-

trypsinogen does not exclude the possibility of a reaction between DFP and an amino acid residue in chymotrypsin. However, it is evident that a reacting amino acid residue must exist in some special linkage or configuration in the protein. It is plausible that this special configuration causes the protein to be an enzyme. The possibility does exist that an unrecognized amino acid or other group occurs in chymotrypsin, but if so it is labile to acid hydrolysis.

It is remarkable that the oxidation of α-chymotrypsin with an excess of periodate results in protein which still retains approximately one-half of its activities. Furthermore, a crystalline protein has been isolated in good yield from the oxidized system.[7] The protein on superficial examination appears to differ considerably from chymotrypsin, and will be studied and reported on later. Goebel and Perlmann (17) have shown that in the periodate oxidation of bovine serum albumin certain amino acids are destroyed and the absorption spectrum and electrophoretic mobility are changed. Prolonged oxidation caused a loss in serological reactivity. However, these effects were observed after prolonged contact with periodate, whereas the altered α-chymotrypsin was recovered after relatively brief exposure (1 hour). It thus appears that oxidized and still active chymotrypsin is a definite entity which can be crystallized.

Since terminal threonine was proved not to be the site of attachment for the diisopropyl phosphate in the inhibition reaction, further investigations, aimed at discovering the identity of this active group in chymotrypsin, are in progress.

SUMMARY

1. The isopropyl groups as well as the phosphate of diisopropyl fluorophosphate (DFP) were found to have been introduced into the crystalline inert protein resulting from the treatment of α-chymotrypsin with DFP. Two isopropyl groups were introduced per mole of enzyme.

2. No fluorine was found in the inert protein. Furthermore, acid (apparently HF) was formed during the inhibition reaction in the proportions of approximately 1 mole per mole of α-chymotrypsin. Therefore, DFP reacts with this enzyme by a condensation reaction with the liberation of the fluorine as HF.

3. The crystalline, inhibited protein had the same amino nitrogen content and electrophoretic mobility as α-chymotrypsin. There is thus no evidence that either an amino or a carboxyl group reacted with the DFP.

4. None of the amino acids known to occur in chymotrypsinogen, even when used in relatively large amounts, interfered with the inhibition of α-chymotrypsin by DFP. Furthermore, an acid hydrolysate of chymo-

[7] Unpublished results.

trypsinogen (after neutralization) likewise did not interfere with the inhibition reaction.   This does not mean that DFP does not react with an amino acid residue of chymotrypsin.   Such a reaction is quite possible, but must then depend on some special configuration in the protein, presumably one by virtue of which the protein is an enzyme.

5. Contrary to preliminary results, DFP did not react with a terminal threonine of α-chymotrypsin.   This terminal threonine was shown to be due to a small amount of impurity in the α-chymotrypsin.   The impurity could be removed from the enzyme by treatment with DFP.   It was isolated and examined.   After crystallization, inhibited chymotrypsin is free from this impurity.

6. Relatively large amounts of tetraethyl pyrophosphate inhibited α-chymotrypsin at a slow rate and trypsin at a still slower rate.

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RELATIONS BETWEEN INSULIN AND PITUITARY HORMONES IN AMINO ACID METABOLISM

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In a previous communication (1) evidence was presented to support the conclusion that insulin acts to accelerate the rate at which proteins are synthesized from free amino acids. This evidence was based on the fact that there is a correlation between the rates at which the individual amino acids are removed from the blood after insulin and the relative molecular proportions of these same amino acids in a representative body protein such as skeletal muscle. In order to extend these studies and fit them into a more comprehensive scheme, a study was made of the interrelations between insulin and the growth and adrenocorticotropic hormones of the anterior pituitary as they influence the metabolism of the amino acids. The purpose of the present paper is to present the results of these experiments.

EXPERIMENTAL

Experiments were performed on normal and alloxan-diabetic adult female dogs, maintained on a diet of laboratory chow, supplemented twice a week with horse meat. The animals were fasted 18 to 24 hours before an experiment. After a control sample of blood was taken, either growth hormone (GH) or adrenocorticotropic hormone (ACTH)[1] was injected intraperitoneally. The hormones were dissolved in 0.001 N sodium hydroxide in 0.9 per cent sodium chloride, and given in a dose of 10 mg. per kilo of body weight. Blood samples, in amounts of 35 ml., were drawn in oxalated syringes at selected intervals thereafter. Neutralized tungstic acid filtrates of these blood samples were prepared immediately and used for the analysis of the amino acids. The amino acids were determined by microbiological assay. The specific methods were the same as those reported previously (1). Only the natural L forms of the amino acids were determined by these methods; therefore all data presented herein are for those forms only.

Experimental diabetes mellitus was induced in dogs, previously fasted

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for 3 days, by the single, rapid, intravenous injection of alloxan monohydrate in a dose that varied from 60 to 75 mg. per kilo of body weight. The dose of 75 mg. was often followed by death in a diabetic-uremic state. Much better preparations were obtained from the use of slightly lower doses. Before use in an experiment these dogs were allowed to recover from the acute effects of the alloxan for a period of at least 2 weeks. By that time the diabetes had become stabilized and the animal was eating well. These dogs were maintained on a mixture of regular and protamine zinc insulin.[2] All insulin was withdrawn for a period of at least 48 hours before an experiment.
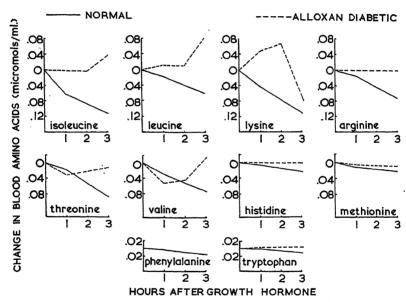
FIG. 1. The changes in blood concentrations of the ten essential amino acids after growth hormone in the normal and the alloxan-diabetic dog. The dose of the hormone was 10 mg. per kilo of body weight.

Results

Experiments with Growth Hormone

Simultaneous changes in the blood concentration of the ten essential amino acids were followed in normal and diabetic dogs for a period of 3 hours after GH injection. The results of one pair of such experiments are presented graphically in Fig. 1. Change in blood concentration is

[2] The author is grateful to Eli Lilly and Company for the supplies of insulin used in this study.

plotted either above or below the control value. The normal dog had a fasting blood glucose of 75 mg. per cent; the diabetic dog had a fasting blood glucose of 280 mg. per cent. It is striking to note the marked difference in response of the blood amino acids to GH in the normal and diabetic dogs. In the normal dog the blood level of each amino acid was depressed. However, in the diabetic animal this was not the case in all instances. There was either essentially no change at all, a fall of short duration followed by a rise to control levels or above, or, as in the case of isoleucine, leucine, and lysine, a definite rise in blood concentration. Preliminary experiments had established the fact that after this dose of GH the fall in blood concentration of all amino acids persisted for at least 6
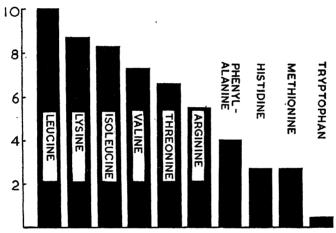
FIG. 2. The relative molecular proportions of the ten essential amino acids in the protein of dog skeletal muscle.

hours. Therefore the response of isoleucine, leucine, and lysine was certainly abnormal, and that of threonine and valine as well, because the preliminary fall in their concentrations was of such short duration.

There is sufficient evidence to allow the statement that GH causes an acceleration of the rate of protein synthesis in the tissues (2). Therefore if the amino acids that disappear from the blood after GH are going into the reactions of protein synthesis, those that appear in increased quantity in the blood of the diabetic after GH must be the reflection of the reversal of the synthetic reactions, namely protein breakdown. It appears from these data that GH, in the presence of insulin, is an agent of protein synthesis, whereas in the absence of insulin, it is an agent of protein breakdown.

In Fig. 2 are presented the relative molecular proportions of the ten

essential amino acids in dog skeletal muscle, which represents the largest single protein mass in the body. Leucine, whose molar concentration was the highest, was given a value of 10, and the concentrations of the other amino acids compared to it on the basis of their relative molecular proportions. By comparison of this graph with the curves for blood amino acid of the normal dog in Fig. 1, it is apparent that under the influence of GH there is, in general, a good direct correlation between the relative rates of fall of the individual amino acids in the blood and the relative molecular proportions of those same amino acids in the protein. Thus it appears that each amino acid is removed from blood in amounts necessary to meet its concentration requirements in the proteins being synthesized. This same relation was found to obtain after the administration of insulin to the dog (1). Therefore these data with GH, a known agent of protein synthesis, strengthen the view that insulin is likewise concerned in the synthesis of body protein.

Experiments with Adrenocorticotropic Hormone

In these experiments the simultaneous change in blood concentrations of three amino acids was followed after the injection of ACTH. Leucine, valine, and histidine were chosen for study because they represent amino acids that are in high, intermediate, and low concentration, respectively, in the protein of skeletal muscle. This is seen by reference to Fig. 2.

In the first type of experiment ACTH was given after a control sample, and the blood concentrations of the three amino acids were followed for a period of 6 hours thereafter. In Fig. 3 are shown the results of such an experiment. It is evident that there was a marked elevation in the blood concentration of all three amino acids. Furthermore there was a direct correlation between the rate of elevation of blood level of each amino acid and the relative proportions of the amino acids in the protein. Leucine, in highest relative proportion in the protein, showed the highest rate of elevation in the blood. Valine, in intermediate concentration in the protein, showed an intermediate rate of elevation in the blood. Histidine, in relatively low concentration in the protein, showed the lowest rate of elevation after ACTH.

In the second type of experiment the dog was first given an intravenous injection of regular insulin in a dose of 2 units per kilo of body weight. At the end of an hour ACTH was given and the blood amino acids followed until the end of the 3rd hour. In Fig. 4 are shown the results of such an experiment. Change in blood concentration, below the control value, is plotted against time in hours. It is seen that the insulin caused a marked fall in the blood level of all three amino acids during the 1st hour. The administration of ACTH then caused a temporary reversal of

the insulin effect, as is seen by the elevation of the blood levels of the amino acids after the ACTH. In this experiment both the rate of fall in concentration of blood amino acid after insulin and the degree of the reversal of the insulin effect with ACTH showed a direct correlation with the relative molecular proportion of each amino acid in the protein. Thus
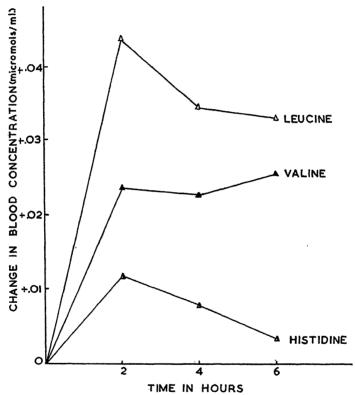

Fig. 3. The effect of ACTH on the blood concentration of leucine, valine, and histidine in the normal dog. The dose of ACTH was 10 mg. per kilo of body weight.

leucine, in high concentration in the protein, showed both the steepest fall after insulin and the greatest rise after ACTH. Valine, in intermediate concentration in the protein, showed both an intermediate rate of fall after insulin and rate of rise after ACTH. Histidine, in relatively low concentration in the protein, showed both the lowest rate of fall after insulin and rate of rise after ACTH.

This proportionality appears in still another instance. Reference to

Fig. 1 will show that, in the diabetic, after GH the greatest elevation in blood concentration was in those amino acids (leucine, isoleucine, and lysine) which are in highest concentration in protein, and that the least
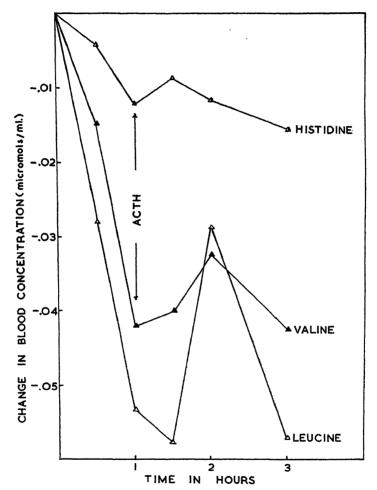
FIG. 4. The effect of ACTH on the blood amino acid response to insulin. The dose of insulin was 2 units per kilo, and that of ACTH 10 mg. per kilo of body weight.

change in blood concentration was in those amino acids (histidine, methionine, and tryptophan) which are in lowest concentration in protein. Thus if fall in the concentration of blood amino acid after GH and insulin in the normal connotes an accelerated rate of protein synthesis, and rise

in blood amino acid concentration after ACTH in the normal and GH in the diabetic connotes an accelerated rate of protein breakdown, it would seem that the rate at which each amino acid is taken into the protein synthetic reaction, or liberated from the protein during its breakdown, is dependent on the concentration of the amino acid in the tissue proteins.

DISCUSSION

Mirsky (3) has studied changes in protein metabolism by following the rate of accumulation of blood urea in the nephrectomized dog. Using an anterior pituitary extract rich in growth properties, he showed that, with the pancreas intact, the extract caused an acceleration of the rate of protein synthesis. With the pancreas removed the same extract caused an acceleration of the rate of protein breakdown. From this Mirsky concluded that the growth hormone of the anterior pituitary requires insulin for its protein synthetic function. Certainly the experiments with purified GH, presented in this paper, are in agreement with this view.

Young (4) has shown that the prolonged administration of anterior pituitary extract will cause typical diabetes mellitus in adult dogs and rats. Sufficient evidence has now accumulated to allow the conclusion that both GH and ACTH, which are present in such extracts, are diabetogenic. Milman and Russell (5) have observed that when purified GH is given to either mildly or severely diabetic rats there is a significant rise in blood glucose concentration. The same sort of phenomenon has been presented in this paper in the case of the blood amino acids in the alloxan-diabetic dog. The diabetogenic effect of GH is further illustrated by the clinical course of the alloxan-diabetic dog following GH administration. It invariably causes the death of the dog within 12 to 48 hours in a state of diabetic acidosis. The time of survival is directly related to the severity of the diabetes at the time of GH administration. It is tempting to speculate that the well stabilized alloxan-diabetic dog is analogous to the animal that has been partially depancreatized. Both have a certain amount of functional islet tissue left. The injection of GH then brings about either the destruction or the effective dysfunction of this remaining tissue, and thus there ensues an intensification of the diabetes.

The mechanism whereby GH is diabetogenic is not certain. Young (4) tends toward the view that it exhausts the islets by forcing them to secrete beyond their capacity. Anderson and Long (6) favor a completely opposite view. In a series of brilliant experiments they made use of an ingenious device for studying the elaboration of insulin by the isolated perfused pancreas. They showed quite conclusively that insulin is normally secreted in response to a high level of glucose in the perfusing blood. If GH is also present in the perfusate, the normal release of insulin is in-

hibited. Thus, whether by exhaustion of the islet cells or by the effective inhibition of release of insulin by the islets, GH negates the normal function of insulin and is therefore diabetogenic.

Conn and his coworkers (7) have been able to produce a temporary diabetes mellitus in human subjects by the repeated injection of ACTH. The mechanism of action of ACTH in this instance is probably an antagonism of insulin at its peripheral biochemical site of action, rather than an inhibition of its liberation from the islets, as in the case of GH. This view is borne out by the experiments with ACTH presented above. The elevation of blood amino acids after ACTH is a typically diabetic response. The effect of the ACTH is to antagonize insulin, as is seen in the second experiment, in which the amino acid response of an exogenous dose of insulin was reversed by ACTH working through its target gland, the adrenal cortex.

Thus the evidence at hand indicates that it is inaccurate to refer to "the diabetogenic hormone" of the anterior pituitary. Certainly we see that at least two hormones of that organ have a diabetogenic function. Although the mechanism of diabetogenic action is different in each case, the end-result is the same. Perhaps other hormones of the pituitary will be shown to have a diabetogenic effect.

SUMMARY

Changes in the blood concentration of a number of individual amino acids have been studied in normal and alloxan-diabetic dogs after the administration of purified growth and adrenocorticotropic hormones. The following observations have been made.

1. In the normal dog, with pancreas intact, GH promotes the synthesis of tissue protein from circulating amino acids. In the diabetic dog, on the other hand, GH promotes the catabolism of tissue proteins.

2. The fact that GH in the diabetic causes an intensification of the diabetes lends support to the conclusion that GH is a diabetogenic factor.

3. The diabetogenic action of pituitary ACTH has been demonstrated in the case of the individual amino acids, and the mechanism of its diabetogenic action discussed.

4. Similarities in the response of the blood amino acids after GH and insulin have been shown to lend support to the view that insulin, as well as GH, causes an acceleration of the rate of synthesis of tissue proteins from free amino acids.

5. Evidence has been presented to show that both the rate of disappearance of the amino acids from the blood during protein synthesis and their accumulation in the blood during protein breakdown are directly related to the concentration of each amino acid in tissue proteins.

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SPECTROPHOTOMETRIC STUDIES

XV. HYDRATION OF MACRO SIZED CRYSTALS OF HUMAN HEMOGLOBIN, AND OSMOTIC CONCENTRATIONS IN RED CELLS*

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WITH THE TECHNICAL ASSISTANCE OF ANNA MAY DYCH, JEAN RANDALL, AND CHARLOTTE GLAUSER

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In preceding reports (1, 2), the effective crystallization of the hemoglobin of man has been described. Macro specimens of small size were seen occasionally in our earlier preparations (1, 2). Recently, we have succeeded in obtaining bipyramidal crystals of human oxyhemoglobin which had long axes up to 1.3 cm. and which weighed up to 0.105 gm. These may perhaps be the largest crystals thus far grown of any protein. In any case, protein crystals of large size are rare, and have been reported previously only for β-lactoglobulin (3).

Hydration of proteins is one of their important properties upon which decisive information is still needed (3, 4). McMeekin and Warner (3) were the first to determine hydration by direct gravimetric measurements on single protein crystals, an accomplishment made possible by the large size of their crystals of β-lactoglobulin. Wet crystals of this protein contained 0.83 gm. of water per gm. of anhydrous protein, an amount several hundred per cent higher than would have been estimated from their data by earlier, indirect methods of calculation (5, 6). The view that the hydration of protein crystals is relatively large is supported by evaluations of the residual water, as deduced from x-ray measurements upon wet and dry crystals (7, 8). With this technique, Perutz (9) has estimated that the hydration of horse methemoglobin crystals varies with their salt content from 0.82 gm. of water per gm. of salt-free protein to 0.69 gm. per gm. in the presence of saturated ammonium sulfate. In this regard, Perutz's conclusions differ from those of McMeekin and Warner (3), who concluded that the degree of hydration was characteristically constant and independent of the salt concentration.[1]

[1] Perutz (9) gives the following values for the density of "salt-free" horse methemoglobin crystals: 1.160 (by flotation in bromobenzene-xylene), 1.242 (by flotation in concentrated salt solution), and 1.270 (air-dried crystals in bromobenzene-

More debatable is the division of the water associated with protein crystals into two fractions, *ordinary* or *"free" water* and what has been designated as *"bound" water*. The molecules of water in the latter fracion are somehow "bound" to the protein and *thereby prevented from exercising their usual solvent properties upon salts*. In the case of horse methemoglobin crystals, Perutz deduced (9) that the amount of such bound water is appreciable, 0.3 gm. per gm. of protein, a value independent of the salt concentration, which agrees with the value calculated by assuming that these particular water molecules are arranged as a monolayer covering the surfaces of the protein exposed to liquid in the crystal structure.[1] In the present paper, data will be supplied on the spectroscopic character, density, hydration, and "bound" water, as directly measured or deduced from the measurements upon individual large sized crystals of human oxyhemoglobin.[2] The bearing of some of these findings upon the interpretation of the osmotic relations between human red cells and plasma will be discussed.

Methods

Crystallization Procedure—Fresh blood, drawn as a therapeutic measure from a polycythemic female subject,[3] was used. A negligible amount of heparin was employed in the syringe, and 75 ml. of a 3.2 per cent solution of sodium citrate dihydrate (slightly more than isotonic) were added as anticoagulant per 400 ml. of the blood. A stroma-free solution of hemoglobin was prepared as previously described (1, 2), and crystallization was accomplished in the refrigerator at a temperature of 4° by dialysis against 2.80 M phosphate buffer, pH 6.8. This buffer solution will be referred to as the *supernatant*.[4] In the initial step, the hemoglobin solu-

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xylene). Consonant with the architecture of the crystal (9, 10), there is a reciprocal relationship of water and salts. The crystal lattice is a *rigid* structure in which the protein (hemoglobin) molecules are arranged in coherent sheets, separated by layers of liquid of crystallization. Dehydration affects only the thickness of the liquid layers; the protein layers appear to be impenetrable by water. Since the spaces in which water and salts can enter are rigidly defined, salts "squeeze out" some of the water as they enter the crystal.

[2] We are indebted to Dr. T. L. McMeekin, Head, Protein Division, Eastern Regional Research Laboratory, United States Department of Agriculture, for certain independent analyses upon our crystals. These data are included with our own.

[3] There is no existing evidence of abnormality of the hemoglobin (or erythrocytes) of individuals with polycythemia vera or Osler's disease. Except for their size, the crystals of hemoglobin conformed to the type previously obtained (1, 2), and the absorption spectra yielded by solutions prepared from the crystallized specimens were identical with those of oxyhemoglobin and derivatives prepared from the blood of normal subjects (*cf.* Table I).

[4] The quantities of salts used in the preparation of the 2.80 M phosphate buffer, pH 6.8 (the supernatant), were approximated from the data of Green (11). The

tions were dialyzed in the refrigerator against the buffer solution previously warmed to 37° (2). At 8 hours, there was no precipitation of hemoglobin, although evident concentration of the solutions had been accomplished. The dialysate was now replaced with fresh cold phosphate buffer. It has been reported (2) that crystallization of nearly the total sac contents occurred in this dialysis within 24 hours. Since that time, we have found that normal bloods are variable in their behavior at this stage, and patience may be necessary. In this particular case, crystallization did not take place until after 3 days of dialysis in the refrigerator, and the crystals were disappointingly small. This led us to continue the dialysis with fresh changes of cold buffer daily over a period of 5 days, followed by further dialysis against the final buffer solution for 7 days. The prolonged dialysis in the refrigerator presumably afforded the proper conditions to allow a remarkable growth of the crystals. At 15 days after the start of dialysis, the crystals were collected, washed in cold fresh 2.80 M phosphate buffer, suspended in this medium, and stored in stoppered flasks in the refrigerator. Periodically, during the course of study, crystals were removed for spectroscopic examination to reveal any alteration which may have occurred.

Preparation of Crystals for Analysis—Individual crystals were removed from the supernatant fluid, the adhering buffer solution was completely drawn off by the frayed edges of torn small pieces of soft filter paper, and the crystal was then weighed quickly with an accuracy of ±0.1 mg. (0.1 to 0.5 per cent). This supplied the original or *wet weight*.

Determination of Protein (Hemoglobin)—The protein content of individual crystals was determined both directly and indirectly. In the direct method, hemoglobin was determined as cyanmethemoglobin in a Klett-Summerson filter photometer (12), calibrated for hemoglobin measurement; so that the photometer readings were directly referable to our spectrophotometrically established constant[5] $\epsilon = 11.5$ at wave-length 540 mμ for cyanmethemoglobin (14, 15). $\epsilon = 11.5$ is referable to the usual accepted value of 0.335 per cent for the iron content of the chromoprotein.

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buffer contained 283.1 gm. of K_2HPO_4 and 160.0 gm. of KH_2PO_4 in 1 liter. The analysis for phosphorus agreed with theory, and was 86.92 mg. per ml. The percentage of phosphate salts by weight = 33.77, and the ratio of phosphate to phosphorus = 443.1/86.92 = 5.098. The density of the supernatant at 25° was 1.3118. Data on density, required in the interpretation of some of the findings (Table III), were also obtained on several buffers of different phosphate concentration, prepared by appropriate dilution from the 2.80 M buffer.

[5] Our ϵ values may be defined as *fractional molar extinction coefficients* (13), referable to an equivalent weight of 16,700 for hemoglobin. Thus, $\epsilon = (1/(c \times d)) \times \log I_0/I$, where the concentration c is expressed in mM per liter, the depth d in cm., the original intensity I_0 is 1.0, and the intensity of transmitted luminous flux I is expressed as a fraction of unity.

When adjusted to our direct analytical value of 0.339 per cent of iron in the crystalline hemoglobins of several species (16), $\epsilon = 11.4$. In the indirect determination, the content of protein was calculated from (*weight of crystal*) — (*weight of water* + *weight of phosphate salts*). The weight of phosphate salts was obtained from the analytical values for phosphorus multiplied by 5.098.[4] Table II shows that close agreement was obtained by the two methods. Hence, hemoglobin may be regarded as the only protein present in the crystals.

Determination of Water—The water content was estimated by difference: (*weight of crystal*) — (*weight of hemoglobin* + *weight of phosphate salts*) = *weight of water*, or by direct analysis, crystal *wet weight* minus *dry weight*. The crystals were dried overnight at 110° in an electric oven, and then in a vacuum desiccator over P_2O_5 at room temperature until constant weight was reached. Flat weighing bottles of 10 ml. capacity, convenient for quantitatively dissolving the dry product for the analysis of phosphorus, were employed. There was agreement between the direct and indirect evaluations of the water content (Table II).

Determination of Phosphate Salts—Of several optional procedures tested, two were found to yield consistent and presumably reliable results for phosphate in material of this type. In each, phosphorus was determined by the Fiske and Subbarow technique (17), modified according to Lohmann and Jendrassik (18), with photometric comparison against standards of inorganic phosphate in the Evelyn photoelectric apparatus (19) with the red (660 mμ) filter. In one procedure (Experiments 3 and 6, Table II), the phosphates were leached out from the oven-dried crystals with three successive extractions with 5 ml. quantities of boiling redistilled water. Dr. McMeekin regarded the extraction of phosphates by this means as complete. There is a suggestion in our own data (Experiment 3, Table II), verified by subsequent analyses after wet incineration of the extracted crystal, that approximately 2 per cent of the total phosphorus had not been removed from the crystal. In the other procedure (Experiments 2, 4, and 5, Table II), phosphorus was determined on solutions prepared after wet incineration. The hemoglobin crystal was digested in micro-Kjeldahl flasks with 1 ml. of the incineration mixture (5 parts of 2 N H_2SO_4 to 1 part of 2 N HNO_3) until SO_3 fumes were formed. Owing to the resistance of hemoglobin to complete digestion, the procedure was twice repeated, once with a further addition of 1 ml. of the incineration mixture, next with 1 ml. plus 5 drops of H_2O_2. The final clear digest was cooled, 10 ml. of redistilled water were added, and the solution was boiled for about 10 minutes. The solution was then transferred to a 25 ml. volumetric flask containing 0.8 ml. of 2 N NaOH (for neutralization to the desired point), and diluted to volume with redistilled water. Small aliquots were

compared against a standard of inorganic phosphate containing 26 γ of phosphorus. In reading the standard (not subjected to digestion) 100 per cent transmittancy was set for a blank of redistilled water, whereas for reading the unknown 100 per cent transmittancy was adjusted to a water blank which had been passed through the digestion procedure.

Determination of Density—An approximate evaluation of density was obtained by flotation of the crystals in phosphate buffer solutions of different concentration,[4] and the exact determination was made by flotation in bromobenzene-xylene mixtures at 25° (3).
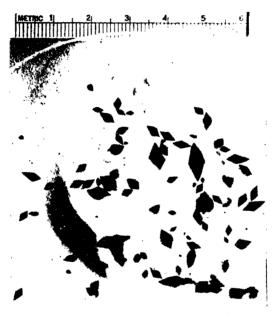
Fig. 1. Photograph of a group of the macro crystals of human oxyhemoglobin, at random orientation in 2.8 M phosphate buffer, together with cm. scale.

Results

Fig. 1 is a photograph of a selected group of the large crystals of human oxyhemoglobin, suspended in the supernatant phosphate buffer. In contrast to the micro crystals (1) these were rather hard and brittle, opaque (owing to their size), dark garnet in color, and with sharply faceted, glistening sides. Like the micro crystals (2), they could be converted in the crystalline state into isomorphous met- and cyanmethemoglobin. The crystalline appearance remained unaltered upon dehydration, particularly

when accomplished by immersion in benzene (3). In this medium some of the crystals have now been preserved for about 8 months.

Table I shows spectrophotometric data which serve to identify the

TABLE I

Spectrophotometric Identification of Pigment Species in Macro Crystals of Human Hemoglobin

Specimen No.	Nature of preparation	Time*	Total pig-ment†	ϵ values‡			Composition of sample§	
				Maxi-mum, 578 mμ	Mini-mum, 562 mμ	Maxi-mum, 542 mμ	HbO$_2$	MHb
		days	*mM per l.*				*per cent*	*per cent*
1	Standard HbO$_2$ (*cf.* (1, 2))			15.34	8.50	14.61	100.0	0.0
2	Standard MHb, pH 7.2 (*cf.* (2))			4.38	4.35	6.70	0.0	100.0
3	Original microcrystalline ppt.	3	0.0850	15.38	8.46	14.60	99.5–100.0‖	0.5–0.0‖
4	Macro crystals (freshly prepared)	15	0.0924	15.15	8.43	14.47	98.3	1.7
5	Macro crystals (preserved in refrigerator)	30	0.0798	14.81	8.31	14.27	95.4	4.6
6	" "	61¶	0.0890	14.56	8.22	14.00	92.7	7.3
7	" "	118¶	0.0765	13.89	8.00	13.68	87.5	12.5

* Days after start of dialysis.

† Determined as cyanmethemoglobin, with $\epsilon = 11.5$ at wave-length 540 mμ. Total pigment was also determined as deoxygenated hemoglobin (on aliquots treated with dithionite, $Na_2S_2O_4$), with $\epsilon = 13.55$ at wave-length 555 mμ (1, 2). The concentration values are those of the dilute solutions, adjusted to pH 7.2, upon which spectrophotometry was performed in the usual 1 cm. depth cuvette.

‡ See foot-note 5.

§ As deduced by the method of Austin and Drabkin (13, 20). The presence of MHb in Experiments 5, 6, and 7 was verified by its reactions with cyanide (conversion to cyanmethemoglobin) and dithionite (reduction to deoxygenated hemoglobin).

‖ Essentially unchanged HbO$_2$ (2, 21).

¶ Period during which most of the measurements relative to the hydration of the crystals were performed.

character of the pigment during the period of study. In the fresh preparation the crystals were of essentially unchanged oxyhemoglobin (Specimens 3 and 4). Suspended in the supernatant solution, and stored in stoppered flasks in the refrigerator, they kept well. The only change found

was a slow partial conversion to met- or ferrihemoglobin (Specimens 5 to 7). The rate of this autoxidative change was appreciably slower than that observed with micro crystals prepared in the presence of $(NH_4)_2SO_4$, pH 5.9 (1), and somewhat slower than that of micro crystals prepared with 2.8 M phosphate buffer, pH 6.8 (2). Most of the measurements of the hydration and composition were performed during the period when the material contained 93 to 88 per cent of oxyhemoglobin and 7 to 12 per cent of methemoglobin (Specimens 6 and 7). The partial oxidation of the hemoglobin had no influence on the relative content of water, total hemoglobin pigment, and salts in the crystal. This could be inferred from the work of Perutz (9), but was verified by us in similar analytical findings at different percentage levels of methemoglobin. That no "denatured" hemoglobin was formed on standing was deduced from the agreement of values for total pigment measured both after conversion to cyanmethemoglobin and to deoxygenated or ferrohemoglobin (see the foot-notes to Table I).

From the data recorded in Table II (and similar results not presented) the composition of individual crystals of human hemoglobin was 57, 35, and 8 per cent by weight respectively of hemoglobin, water, and phosphate salts. The mean values of direct analyses, with their standard deviations (see the foot-notes to Table II), were as follows: hemoglobin (iron basis), 57.3, s.d. = 1.35 ± 0.43; water, 35.0, s.d. = 0.59 ± 0.21; phosphate salts, 7.92, s.d. = 0.12 ± 0.04.

Table II also furnishes calculated values for hydration (total water per gm. of protein), percentage by weight of phosphate salts in the water of the crystal, and the percentile ratio of phosphate concentration in crystal water to that in the supernatant liquid. Since the individual analyses are in close agreement, the mean values for these quantities may be calculated from the mean percentages of water, hemoglobin, and phosphate given above, with the following results: *hydration per gm. of protein* = 0.61 gm., *percentage of phosphate in crystal water* = 18.45 per cent, and *percentile ratio of phosphate in crystal water to phosphate in supernatant* = 54.6 per cent.

Under our conditions, complete equilibration of the crystal water with the supernatant salt solution was assured, and the concentration of phosphates in the crystal water should have been identical with that in the 2.80 M buffer. The alternative explanations of the low phosphate concentration in the crystal water may be considered: (1) An appreciable fraction of the total water in the crystal was not free to dissolve salts. (2) The phosphates were dissolved in the total water, but only a limited amount of the salts had been able to penetrate the crystals. An attempt was made to test both hypotheses by a comparison, presented in Table III, of two calculated values for the density of individual crystals with the deter-

mined density. The calculations were performed as explained in the foot-notes to Table III. The density of the crystals, determined by flotation in bromobenzene-xylene mixtures, was 1.2440 (mean of values in Column 7, Table III). By the first method of calculation, in which the liquid of the crystal is assumed to be composed of 2.80 M phosphate

TABLE II

Analytical Data on Individual Hemoglobin Crystals

In Experiment 2, hemoglobin and phosphorus content were determined; water content was obtained by difference. In Experiments 3 and 6, water and phosphorus content were determined; protein (hemoglobin) was obtained by difference.

	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6*
Weight of crystal, *mg.*	46.9	25.8	29.7	30.4	11.50
Protein (Hb), *mg.*	26.50	14.70			6.54
" % *by weight*	56.5	57.0	57.3†	57.3†	56.9
Phosphorus, *mg.*	0.729‡	0.412 (0.402)§	0.464‡	0.456‡	0.178§
Phosphate salts, *mg.*‖	3.72	2.10 (2.05)	2.37	2.32	0.91
" " % *by weight*	7.92	8.14 (7.94)	7.98	7.63	7.91
Water, *mg.*	16.68	9.0			4.05
" % *by weight*	35.6	34.9	35.0¶	35.0¶	35.2
" *gm. per gm. Hb*	0.63	0.61			0.62
Phosphate in water (liquid) of crystal, % *by weight*	18.24	18.92	18.56	17.90	18.35
Ratio (× 100), concentration of phosphate in crystal water to phosphate in supernatant**	53.8	56.0	54.9	53.0	54.3

* Analytical data obtained by T. L. McMeekin.

† Mean of analytical values for hemoglobin (iron basis, see "Methods"); s.d. = 1.35 ± 0.43, where s.d. = $\sqrt{\Sigma\ d^2/n - 1}$ and the plus-minus value is the standard error of the standard deviation = (s.d.)/$\sqrt{2n}$.

‡ Determined following wet incineration of crystal.

§ Leached out from oven-dried crystal. Values in parentheses obtained before correction for non-extracted phosphate; in Experiment 6 extraction regarded as complete.

‖ Calculated from phosphorus × 5.098 (see foot-note 4).

¶ Mean of analytical values for per cent by weight of water; s.d. = 0.59 ± 0.21.

** See foot-note 4.

buffer solution plus salt-free water, the average of the calculated densities (Column 6, Table III) is 1.2462. This is considered to be in agreement with the determined value of 1.2440, since, at present, some uncertainty enters into such calculations owing to the use of an imperfectly established value (4, 6) of 0.750 for the partial specific volume of the protein (Column 3 and foot-notes, Table III). On the other hand, by the second method of calculation, in which the liquid of the crystal is assumed to be

TABLE III

Density of Individual "Wet" Hemoglobin Crystals at 25°

The calculated values are based on data in Table II.*

Experiment No. (1)	Composition (2)	Partial specific volume† (3)	Weight fraction in crystal‡ (4)	Partial volumes in crystal and specific volume (V)§ (5)	Density Calculated ($1/V$) (6)	Density Determined (7)
2, a	Hb	0.750	0.5650	0.4238		
	2.80 M phosphate	0.762	0.2349	0.1790		
	Water (0.563)	1.003	0.2001	0.2007		
	Sum			0.8035 (V)	1.2445	1.2425–
						1.2540‖
2, b	Hb	0.750	0.5650	0.4238		
	1.333 M phosphate, d 1.1562¶	0.865	0.4349	0.3762		
	Sum			0.8000 (V)	1.2500	
3, a	Protein (Hb)	0.750	0.5698	0.4274		
	2.80 M phosphate	0.762	0.2410	0.1836		
	Water (0.542)	1.003	0.1892	0.1898		
	Sum			0.8008 (V)	1.2488	
3, b	Protein (Hb)	0.750	0.5698	0.4274		
	1.390 M phosphate, d 1.1629¶	0.860	0.4302	0.3700		
	Sum			0.7974 (V)	1.2541	
5, a	Hb	0.750	0.5730	0.4298		
	2.80 M phosphate	0.762	0.2260	0.1722		
	Water (0.572)	1.003	0.2003	0.2009		
	Sum			0.8029 (V)	1.2455	
5, b	Hb	0.750	0.5730	0.4298		
	1.304 M phosphate, d 1.1529¶	0.867	0.4263	0.3696		
	Sum			0.7994 (V)	1.2509	
6, a	Protein (Hb)	0.750	0.5687	0.4265		
	2.80 M phosphate	0.762	0.2343	0.1785		
	Water (0.559)	1.003	0.1970	0.1976		
	Sum			0.8026 (V)	1.2460	1.2424**
						1.2435**
						1.2460**
6, b	Protein (Hb)	0.750	0.5687	0.4265		
	1.342 M phosphate, d 1.1573¶	0.864	0.4312	0.3726		
	Sum			0.7991 (V)	1.2514	

* Alternative methods are used for the calculation of the density of the crystal. In Experiments *a*, the crystal liquid is assumed to be composed of 2.80 M phosphate buffer solution plus phosphate-free water. The value in parentheses after water (Column 2) is the fraction of the total crystal water which is inoperative in dissolving salt. In Experiments *b*, the crystal liquid is assumed to be phosphate buffer solution of the concentration which would be present if the phosphate salts were dissolved in the total water of the crystal.

† Partial specific volume = 1/(density). Density of protein taken as 1.333 (4, 6); determined density of the 2.80 M phosphate (the supernatant) at 25° = 1.3118 (see foot-note 4); the density of water at 25° = 0.9970.

TABLE III—*Concluded*

‡ Weight fraction of protein = (weight of protein (Hb))/(weight of crystal). Weight fraction of 2.80 M phosphate = ((weight of phosphate)/0.3377)/(weight of crystal) (see foot-note 4). Weight fraction of water = (weight of total water in crystal minus weight of water in 2.80 M phosphate in crystal)/(crystal weight).

§ Values in Column 3 multiplied by corresponding values in Column 4.

‖ Approximate values determined by flotation in several phosphate buffer mixtures at 25°. Crystal just sank in 2.128 M phosphate buffer of density 1.2425. Crystal floated in 2.240 M phosphate buffer of density 1.2540.

¶ Density value extrapolated from analytical data on density of phosphate buffer solutions of different concentration, prepared by dilution from original 2.80 M buffer.

** Values obtained by T. L. McMeekin on individual crystals by flotation in bromobenzene-xylene mixtures at 25° (3). The analytical value of 1.2424 was regarded by McMeekin as probably the best.

phosphate buffer of the concentration which would be obtained if the salts which had penetrated had been dissolved in the total water, the individual calculated values are consistently too high (Column 6, Table III), with a mean of 1.2516. Hence, we believe that the finding of low phosphate concentration in the crystal water is cogent evidence for the existence in the crystal of water inoperative in dissolving salts.

In the crystals, this fraction of "bound" water is of appreciable size, as is evident from the calculated values in Column 2, Table III. With our mean values of 7.92 per cent (by weight) of phosphate in the crystal, 33.77 per cent of phosphate in the 2.80 M phosphate buffer,[4] 35.0 per cent of water in the crystal, and 0.61 gm. of total water per gm. of protein, the following may be calculated: *fraction "bound" of total water in crystal* = 0.556, and *gm. of "bound" water per gm. of hemoglobin* = 0.339. The latter value, derived from direct measurements on our macro crystals of human hemoglobin, is in essential agreement with that of 0.3 gm. per gm. of protein, deduced by Perutz from x-ray data on crystalline horse methemoglobin (9).[1]

As in the case of β-lactoglobulin (3), about 75 per cent of the total water in our hemoglobin crystals is lost upon exposing them to air for 14 to 18 hours at room temperature. Since 56 per cent of the crystal water is "bound," some of the water from this fraction must be lost. If it may be assumed that all the "free" water leaves the crystal first, it may be calculated that, in addition, about 50 per cent of the "bound" water is lost on drying in air.

DISCUSSION

The water adsorbed on proteins is currently believed to be present in multilayers (22, 23). According to Pauling (24), the amount of water in the first adsorbed monolayer of many proteins agrees with that calculated

for a mole per mole combination with polar groups in amino acid residues, exclusive of the carbonyl and imido groups of the peptide linkages. In the case of horse methemoglobin, the amino acid composition of which is established (25, 26), it may be calculated that per 2 molecules (in the unit cell), each with a molecular weight of 66,800, there are about 600 moles of such polar groups, effective in combining with an equal molar quantity of water. 600 moles of water are approximately one-fourth of 2223 moles per 2 moles of hemoglobin, calculated from 0.3 gm. of "bound" water per gm. of protein. Hence, if Pauling's hypothesis is correct, four monolayers of "bound" water may be present, with only one of them in direct chemical union with the protein. A similar relationship should hold for human hemoglobin, since the total number of polar amino acid side chains is probably not materially different from that in horse hemoglobin (27, 28). Our deduction that 50 per cent of the "bound" water is lost easily suggests either that two of the four monolayers are more firmly bound or that other hydrophilic groups, besides those postulated (24), are operative. If, in the hemoglobins, the carbonyl and imido groups are also capable of combining with water, the total molar concentration of hydrophilic side chains would be doubled (24). This would be consonant with our observations.

Inferences as to total hydration in the dissolved state cannot be made safely from the findings in the crystal (4). On the other hand, we believe that the value for "bound" water in the crystal is applicable to hemoglobin in the red cells. Several facts support this view-point. (1) In contrast to total crystal water, the "bound" water is invariant with the concentration of salt (9). (2) The concentration of hemoglobin in the red cell is very high, indeed close to the maximum possible for the volume relationships of the cell and its major constituents (29). (3) As revealed by the method of x-ray scattering at low angles (30), in the red cells, but not in dilute solutions, the molecules of hemoglobin appear to be in a state of organization and orientation.

Osmotic Equilibrium in Red Cells and Plasma—Human erythrocytes contain 340 gm. of hemoglobin (29) and 715 gm. of water (a mean value (31, 32)) per liter. The proposal that the value, found by us in crystals, for water inoperative as a solvent (0.339 gm. per gm. of hemoglobin) is applicable to the human red cells means that, per liter of cells, 115 gm. of water (from 340×0.339) or 16 per cent of the total 715 gm. are restricted, and only 600 gm. are free to exhibit their normal properties. This must have an important consequence on the osmotic relationship of red cells and plasma.

The cells are recognized to be in osmotic equilibrium with the plasma (33, 34). Nevertheless, a troublesome discrepancy has existed between the total osmotic concentrations in human erythrocytes and plasma, cal-

culated from their known compositions in milliequivalents per liter of solution, illustrated in the ionic diagrams,[6] A to C, Fig. 2. In Diagrams D
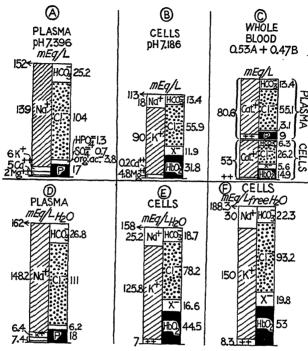

FIG. 2. Ionic diagrams of arterial human plasma, red cells, and whole blood. In Diagrams A, B, and C concentrations are per liter of fluid; in Diagrams D and E per liter or per kilo of water; in Diagram F per liter or per kilo of "free" (osmotically effective) water. See foot-note 6 for data used in the construction of the diagrams.

and E, the same data are given in milliequivalents per liter or per kilo of water. When appropriate corrections to di- and polyvalent ions in the

[6] The values in the ionic diagrams are applicable to the arterial blood at 37° of an adult resting male, with hemoglobin of whole blood = 160 gm. per liter (15), hemoglobin of red cells = 340 gm. per liter (29), oxygen saturation = 98 per cent (21), pCO_2 = 41.5 mm. of Hg, solubility coefficient, α, for CO_2 = 0.522 in plasma, 0.45 in cells, and 0.489 in whole blood, and the fraction of plasma and cells = 0.53 and 0.47 respectively. Water is taken as 938 gm. per liter in plasma and 715 gm. per liter in cells (31, 32). The values used for total CO_2, TCO_2, are 27.0 mM per liter in plasma and 13.39 (or 13.4) mM in cells and for dissolved CO_2, DCO_2, 1.272 mM in plasma and 1.098 mM in cells. The pH is calculated from pH = pK^* + log ([TCO_2 − DCO_2]/[DCO_2]), where pK^* = 6.09 for plasma and 6.03 for cells. These pK values, rather than the usual 6.10, were kindly supplied by Dr. Richard B. Singer. Their

heterogeneous system of whole blood are applied (Diagram C, Fig. 2) and the non-ionized components are included, the summation of the osmotically active components in the plasma fraction (0.53 of the whole blood), $\Sigma(\text{mOsM})_p$, = 159.8, while in the cells (0.47 of the whole blood), $\Sigma(\text{mOsM})_c$ = 92.0. The ratio of these quantities = 1.737. On the other hand, the ratio of water in the two compartments, H_2O_p/H_2O_c = $(938 \times 0.53)/(715 \times 0.47)$ = 1.479. Thus, the osmotic discrepancy, evaluated by the factor 0.852 (from 1.479/1.737), becomes apparent. If, now, our corrected value of 600 gm. for "free" water per liter of cells is employed, H_2O_p/H_2O_c = $(938 \times 0.53)/(600 \times 0.47)$ = 1.762. The discrepancy is removed. The corrected ionic composition of red cells (in milliequivalents per liter of "free" water) is furnished in Diagram F, Fig. 2. The slight overcorrection (1.762/1.737 = 1.014) may well be due to the presence of some unevaluated "bound" water in the plasma. We regard this as supporting evidence of the applicability to the red cells of our experimental value for "bound" water in the crystals of human hemoglobin. Our value (16 per cent) for osmotically inoperative water in erythrocytes is intermediate between high values approaching 30 per cent (35, 36) and the value of 8 per cent, the highest found by Hill (33) with the vapor pressure method. Hill concluded that bovine red cells contained only negligibly more "bound" water than bovine plasma.

It should be pointed out that the presence of "bound" water is pertinent also in considerations involving the Donnan theory (37).

SUMMARY

By prolonged dialysis at 4° against 2.8 M phosphate buffer, pH 6.8, unusually large crystals of human oxyhemoglobin were obtained.

The mean analytical composition of single crystals was as follows: hemoglobin 57, water 35, and phosphate salts 8 per cent by weight. The minimal density of the crystals was 1.2424.

The concentration of phosphate salts in the water of the crystal was only 54.6 per cent of that in the supernatant phosphate buffer after thorough equilibration with the crystal.

use is empirical but affords adjustment for the presence of carbamino CO_2, CCO_2, = 0.5 mM per liter in arterial plasma and 2.3 mM in cells (by calculation), and allows correct evaluation of HCO_3^- from TCO_2 and DCO_2. In the ionic diagrams CCO_2 is included with the protein anions, P or HbO_2. The calculated pH values agree closely with the data of Dill et al. (32), whose values for inorganic ions (except HCO_3^-) were used after adjustment to the above conditions. The value of 31.8 milliequivalents per liter of hemoglobin in the cells is derived from unpublished titration data of R. B. Singer and A. B. Hastings on human hemoglobin, prepared by our method. X^- in cells represents undetermined anions (largely organic phosphates, etc.).

The hydration of the crystal, calculated from the analytical data, was 0.61 gm. per gm. of protein. Of the total water 55.6 per cent or 0.339 gm. per gm. of protein was "bound" (inoperative in dissolving salt).

The proposal has been made that the value for "bound" water in the crystal is applicable to hemoglobin in the red cell. This hypothesis satisfactorily removes the discrepancy between the total osmolar concentrations of human erythrocytes and plasma, calculated from their respective compositions, and must also be considered in applying Donnan's theory of membrane equilibria.

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SIZE AND DENSITY OF POLYSTYRENE PARTICLES MEASURED BY ULTRACENTRIFUGATION*

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A material currently in use (1) for calibration of electron magnification is a polystyrene latex, characterized by a remarkably high degree of particle uniformity with respect to size and shape. The particles, spherical in shape and stable in water suspension, are in a range suitable not only for electron micrography but for independent determinations of size by sedimentation velocity, viscosity, and light-scattering methods. Consequently, the material offers opportunities for checking the theories underlying measurements of size by these various methods. In the work reported here,[1] studies have been made on the size of the particles estimated from data obtained with the ultracentrifuge. In order to make the calculations of size, studies were made also on the density and state of dispersion of the particles. These accessory data are of interest for comparison with the results previously reported on analogous investigations of the size and density of the influenza (2, 3) and other viruses (4).

Materials and Methods

The polystyrene latex was of Batch 580G, prepared by The Dow Chemical Company, Midland, Michigan, and obtained in water suspension from Dr. Robley C. Williams. An electron micrograph of the material is shown in Fig. 1. The suspension contained about 4 per cent by weight of the particles. Sedimentation velocity studies were made on the particles in 0.9 per cent NaCl, in Ringer's solution, and in 0.06 M phosphate buffer of pH 7.2. There was no evidence of aggregation or precipitation of the particles in these media. Studies on the relation of sedimentation rate to concentration of the particles were made in 0.9 per cent NaCl.

Density of the particles was investigated to provide data for calculation of particle diameter. These studies were made by sedimenting the particles in suitable concentration, 6.66 mg. per ml., in D_2O (3) and in bovine serum albumin (2-4) by the techniques previously described in analogous

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studies on viruses. Solvent density was varied by suitable dilutions of the D_2O or bovine serum albumin with water. In all instances, the content of NaCl was maintained at the constant level of 0.9 per cent. The maximum density obtained with D_2O was 1.092 and that with bovine serum albumin was 1.073.

Sedimentation velocity studies were made in the air-driven ultracentrifuge, carrying a rotor cell of 5 mm. thickness and 4° arc width, turning on a mean radius of 6.5 cm. Photographs of the sedimenting boundary were made on Ansco process film by use of a high pressure capillary mercury arc with the lens and filter arrangements of Svedberg and Pedersen (5).
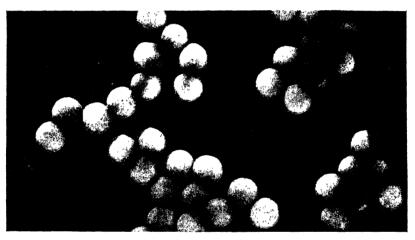
FIG. 1. Electron micrograph of polystyrene latex spheres, shadow-cast with chromium.

Results

For studies on the influence of concentration on sedimentation rate, a series of dilutions of the particles was made with 0.9 per cent NaCl solution. The preparations contained 20, 10, 6.66, 2.5, 0.2, and 0.04 mg. of polystyrene per ml., respectively. In this series of studies, the particles were sedimented at 2890 $\times$ g. The results are illustrated in Fig. 2.

At all of the concentrations down to and including 2.5 mg. per ml., there was a single, exceedingly sharp boundary (Fig. 3), indicative of a high degree of particle uniformity in the suspensions. At concentrations of 0.2, Fig. 4, and 0.04 mg. per ml., there was still sufficient light absorption for photography, but the sedimentation rate was substantially less at 0.2 mg. per ml. than at 2.5 mg. per ml., and at 0.04 mg. per ml. there was a further decrease accompanied by erratic splitting of the boundary.

These last two runs have been excluded in calculations from these data (Fig. 2).

The results of the density studies are shown in Fig. 5. It is seen that when the density of the medium was 1.033 sedimentation still proceeded away from the axis of rotation, but at 1.06 and above the direction was reversed, the particles moving in toward the axis from the cell bottom. The line was drawn by the method of least squares through the data obtained with D_2O, because relatively small viscosity corrections were needed (6), and these data are, therefore, regarded as more reliable than those obtained with bovine serum albumin. The data obtained with bovine
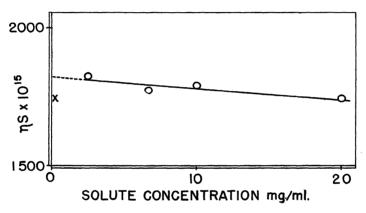
FIG. 2. Relationship between the corrected sedimentation rate (ηS in centimeter-gram-second units) of polystyrene spheres in 0.9 per cent NaCl solution and the concentration of the spheres in the suspension. The point, X, away from the line was not employed in the calculations, since the sedimenting boundary became unstable in this region of concentration of the particles.

serum albumin, however, are in close agreement. The intersection of this line with the zero axis indicates a particle density of 1.053 for the sedimenting particles in the D_2O-H_2O mixtures containing 0.9 per cent NaCl.

The mean value of particle size can be calculated approximately from the data of Fig. 5 as follows

$$\frac{4}{3}\pi r^3(\rho - \rho_S)\omega^2 R = 6\pi\eta r \frac{dr}{dt} \tag{1}$$

$$S = \frac{1}{\omega^2 R}\frac{dr}{dt} \tag{2}$$

$$2r^2(\rho - \rho_S) = 9\eta S \tag{3}$$
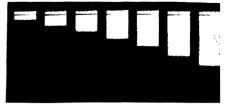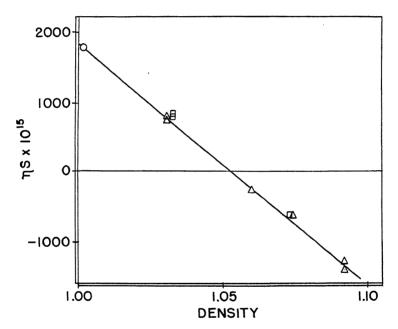
<table>
<tr><td align="center">FIG. 3</td><td align="center">FIG. 4</td></tr>
</table>

FIG. 3. Boundary of the polystyrene spheres sedimenting in 0.9 per cent NaCl at a concentration of 20 mg. of the material per ml.

FIG. 4. Sedimenting boundary of the material of Fig. 3 diluted with 0.9 per cent NaCl to 0.2 mg. of polystyrene per ml.

[[FIG. 5. Relationship between corrected sedimentation rate (ηS in centimeter-gram-second units) of polystyrene spheres and the density of the suspending medium. The concentration of polystyrene was 6.66 mg. per ml. and that of NaCl was 0.9 per cent for all preparations. The density of the suspending medium was adjusted with D_2O in one series of sedimentations ($\triangle$) and with bovine serum albumin in the other ($\square$). The circle is the value of ηS for this polystyrene concentration in 0.9 per cent NaCl taken from the *line* of Fig. 2.

where $4/3\pi r^3$ is the volume of the spherical particles of radius r; $\omega^2 R$ is the centrifugal acceleration at distance $R^{cm.}$ from the axis of the rotor

running at ω radians per second; $6\pi\eta r$ is the frictional resistance to sedimentation of a sphere of radius r through a medium of viscosity η; $S =$ the sedimentation rate per unit acceleration (5); $\rho =$ the density of particle; and $\rho_S =$ the density of the suspending medium.

Clearly, ηS is linearly dependent on ρ_S, and it vanishes when ρ_S is adjusted to ρ. The data bear this out, giving intersection with the zero axis at $\rho_S = \rho = 1.053$. From the first derivative of ηS with respect to ρ_S, it is seen that the magnitude of the slope of this line is

$$\frac{d(\eta S)}{d(\rho_S)} = \frac{2r^2}{9} = \text{slope}$$

a simple measure of the radius squared. Particle diameter, $D = 6\sqrt{-(\text{slope})/2}$. A slight correction must be made in measurement of the slope, because the sedimentation rate is not independent of the concentration of suspended particles. The correction is made by increasing the measured slope by the factor 1820/1790, which is the ratio of ηS extrapolated to infinite dilution (Fig. 5) to its value at 6.66 mg. per ml., the concentration used in these density experiments. When this is done, the calculated sedimentation constant is $1940S$, and the mean particle diameter is 253 mμ with an estimated variation of ± 3 mμ. It is, therefore, highly probable that measurement of a very few images of these spheres in an electron micrograph is sufficient, for most purposes, to establish the magnification.

DISCUSSION

It is difficult to say with precision how closely the particle sizes are grouped about the mean value associated with the sedimenting boundary in the present experiments. It is estimated that the total spread of boundary in Fig. 4 (0.2 mg. per ml. concentration) is not over 2 per cent, some of which is attributable to mechanical and photographic imperfections in the centrifuge arrangement. This figure, 2 per cent, at face value, would reflect only a 1 per cent spread in the diameters of the sedimenting spheres. Furthermore, if some particles sediment substantially more slowly than the main body of the material, their concentration must be considerably less than one-hundredth part of the total, as seen from Fig. 3 which shows the sedimentation on a sample containing 20 mg. of the particles per ml. Light penetrated the region of the supernatant fluid and the air space above the solution with equal ease but, had 1 part in 100 of slower sedimenting particles been present, this quantity should have absorbed as much light here as was absorbed in Fig. 4 when the concentration of sedimenting material was 0.2 mg. per ml. No such critical test

for particles larger than the mean is supplied by these pictures, but any homogeneous component greater than a few per cent could have been seen below the principal boundary of Fig. 4.

The particle diameter calculated from the sedimentation data, 253 ± 3 mμ, was somewhat lower than the value, 2590 ± 25 A, which has been obtained (1) by direct measurements in the electron microscope.

In the present experiments, it was noted that the sedimenting boundary, at low concentrations of the particles (0.04 mg. per ml.), tends to break up in a manner suggestive of instability owing to lack of sufficient density differential at the boundary between the supernatant fluid and the solution below. The low sedimentation rate seen at 0.2 mg. per ml. may be evidence of the onset of this condition. Preparations in which the density of the medium was near 1.05 show multiple boundaries with the higher concentrations of polystyrene particles, in contrast with the single boundaries seen in media of density near 1.0 or 1.1, both of which values are far from the density of the particles. It would appear from this that the effect is indeed one of boundary instability in the ultracentrifuge at these low density increments, and this may explain similar behavior previously observed in low concentrations of both the T$_2$ (7) and the T$_7$ (8) bacteriophages of *Escherichia coli*.

The procedure for measuring the density of particles by sedimentation in the ultracentrifuge has been employed in earlier studies on the properties of viruses. In the study of virus density, it was observed (2, 9–11) that the sedimentation properties of the virus particles changed in solutions of densities varied by solutes of low molecular weight such as NaCl, sucrose, and others, so that the relation between sedimentation rate and density of the suspending medium was not linear. It was supposed (2, 10) that this finding was due to osmotic effects of the solute on the virus, resulting in changes in the water content of the agents, and it was for this reason that the relatively high molecular weight bovine serum albumin was employed (2, 4) for measurement of the wet density of the virus particles. With this material, linear relations were observed. The use of D$_2$O was based on the supposition (3, 12) that this material might freely penetrate the virus particles and, consequently, that measurements of sedimentation rate in solutions would give the dry density of the particles or, perhaps, more correctly, the reciprocal of the partial specific volume. Results have been reported that appear to give weight to these hypotheses.

It will be observed in Fig. 5 that the albumin and D$_2$O curves obtained with polystyrene latex are essentially identical, in contrast with the analogous relations found with influenza virus (2). This finding indicates that little, if any, water is associated with the polystyrene particles in

these solvent mixtures. The value, 1.053, obtained by means of D_2O was not greatly different from that, 1.052, determined by an entirely different method (13). These results with polystyrene particles support the validity of the previous interpretations relative to the wet and dry density of viruses and the theories concerned with the use of bovine serum albumin and D_2O in the determination of these respective properties of virus particles.

SUMMARY

Studies have been made on the size of polystyrene latex particles by means of sedimentation in the ultracentrifuge. Single, sharply sedimenting boundaries were observed in the region of particle concentration of 2.5 to 20 mg. per ml., indicating high uniformity of particle size, and in this region the sedimentation rate was linear with concentration. At lower concentrations the rate diminished and the boundary became unstable. The calculated sedimentation constant was $1940S$.

Particle density was determined by sedimenting the material in D_2O solution which gave the value 1.053. Data in close agreement with those obtained with D_2O were observed with bovine serum albumin. The calculated diameter of the particles was 253 mμ.

The findings with polystyrene latex particles are compared with the results of analogous studies with virus particles.

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THE SEPARATION OF NATURAL ESTROGENS BY COUNTER-CURRENT DISTRIBUTION*

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The development of fluorometric methods (1–4) for the quantitative estimation of estrogens in the small quantities present in human urine has opened the way for improvements in the procedures utilized for the extraction and separation of these compounds. A study of the partition coefficients of estrone, estradiol-17β,[1] and estriol in various solvent systems has been of great value in the development of a simplified extraction procedure. Counter-current distribution of the purified extracts has been found useful for the separation of the three estrogens, for their quantitative estimation, and for the evaluation of the specificity of the extraction procedure.

Methods

Photofluorometric Analysis—Estrogen solutions were analyzed by the method of Bates and Cohen (2) with the following modifications: The samples were dissolved in 0.2 ml. of alcoholic toluene (1 volume of U. S. Industrial Chemicals, Inc., U. S. P., absolute ethanol, and 19 volumes of Baker's c.p. toluene). Heating with 90 per cent (by weight) du Pont sulfuric acid was carried out for 10 minutes in a boiling water bath and after cooling the solution was diluted with 7 ml. of 65 per cent sulfuric acid (du Pont, sp. gr. 1.553 at 20°). The fluorescence was read in a Coleman model 12-A photofluorometer with a Coleman B-2 (436 mμ) lamp filter and a Farrand interference filter No. 1405 (525 mμ) as the photocell filter.

Partition Coefficients—Alcoholic estrogen solution containing approximately 100 γ was pipetted into a separatory funnel and the alcohol was removed under reduced pressure. Equal volumes (25 ml.) of upper and

lower phase mutually saturated were added and shaken for 2 minutes. Each phase was analyzed either directly, if a calibration curve was available, or after evaporation of an aliquot and solution in alcoholic toluene.

Counter-Current Distribution—Partition coefficients of estrogens in several solvent mixtures were determined in the manner described in the preceding section. Systems which showed promise of giving good separation of estrogens were then run on a Craig twenty-four transfer counter-current distribution machine (6) with equal volumes in each layer. At the end of the distribution the contents of each tube were removed and made homogeneous by the addition of ethanol. Aliquots of these solutions were transferred to 19 × 150 mm. Pyrex test-tubes and evaporated to dryness in a water bath with a manifold through which a rapid current of air was drawn. Each tube was then placed on a vacuum line for a few seconds.

When urinary extracts were analyzed, one of three procedures was used: (*a*) the procedure described above; (*b*) the analysis of a single layer; or (*c*) evaporation of the total tube contents to dryness at a pressure less than 1 mm. of mercury and solution of the residues in measured volumes of ethyl acetate. Method (*a*) may be used when at least 200 γ of each estrogen are present, method (*b*) when large amounts of estrogen and small amounts of fluorogenic or chromogenic contaminants are present, and method (*c*) when small amounts of estrogens are present. When method (*b*) is employed, the organic layer is analyzed unless very small aliquots of the aqueous layer suffice.

DISCUSSION AND RESULTS

Photofluorometric Analysis—A typical calibration curve for the three estrogens in alcoholic toluene is shown in Fig. 1. The linearity and high sensitivity should be noted. In pure solution the estrogens may be determined with an accuracy of ±3 per cent. Solvents other than alcoholic toluene may be employed, but all of those which are miscible with sulfuric acid decrease the fluorescence of the estrogens. Methanol and hydrochloric acid markedly quench fluorescence. Toluene gives higher readings than the alcoholic toluene employed, but the pronounced insolubility of estriol in aromatic hydrocarbons renders them useless. Of practical value would be the elimination of solvent altogether. This was tried, but the results were both irregular and irreproducible. The use of Mallinckrodt sulfuric acid in place of du Pont sulfuric acid produces non-linearity of the calibration curves. This effect has also been noted elsewhere.[2] The use of a dilute stock solution (<10 γ per ml.) of estrogens in alcohol or glacial

[2] Private communication from Dr. R. W. Bates.

acetic acid was avoided, for even storage in the cold did not prevent significant changes in the titer within a few weeks.

When the light source is changed, new calibration curves must be constructed since there is variation in the output of the mercury vapor lamps used.

Hydrolysis and Extraction—The hydrolysis of the water-soluble estrogen conjugates is a vexing problem. Preliminary experiments[3] indicated that higher yields of estrogens are obtained after autoclaving pregnancy urine with 15 volumes per cent of 12 N sulfuric acid for 5 to 10 minutes, than after refluxing for 10 minutes with the same concentration of acid. This is in agreement with the findings of Stevenson and Marrian (7) and Van Bruggen (8).
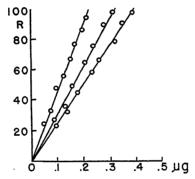
FIG. 1. Calibration curve of estrone (first curve), estradiol-17β (third curve), and estriol (second curve). R is the scale reading of the fluorometer.

Table I summarizes the partition coefficient data which provide the basis for the extraction procedure outlined in Table II. It is of importance to emphasize that complete recovery of the three estrogens is obtained by ether extraction of the alkaline extracts after adjusting the pH of the solution to 9 ± 0.5 (Fig. 2). This is in essence a return to the original method of Cohen and Marrian (9), who saturated the sodium hydroxide extracts with carbon dioxide. Mather (10), too, pointed out that estriol, the most hydrophilic of the three compounds, could be extracted by ether from 0.3 M sodium carbonate. Since the pK values of estrone and estriol are 9.36 and 9.11, respectively (11), it is apparent that lowering the pH to any value less than 7 serves no useful purpose.

Employment of higher pH values increases the purity of the extracted estrogens by the elimination of much of the pigment. Since a large amount

[3] Unpublished data.

of pigment interferes with photofluorometric as well as with colorimetric determinations, any device which will eliminate pigment in whole or in part is of considerable importance.

Counter-Current Distribution—Satisfactory separations with twenty-four transfers may be anticipated when the partition coefficient lies between 0.05 and 20 and when the ratio of the partition coefficients of two neighboring components is approximately 3 or greater.

A tabulation of partition coefficients in biphasic ternary and quaternary solvent systems is given in Table III. Some of the values were determined prior to the development of the fluorometric technique and were measured

TABLE I

Partition Coefficients of Estrogens and Their Application to Extraction

| System | Partition coefficient | | | | | |
| | K^* | | | Per cent estrogen lost† | | |
	E_1	E_2	E_3	E_1	E_2	E_3
Ether-1.5 N H_2SO_4	100	80	50	0.1	0.1	0.1
Ether-saturated $NaHCO_3$	∞	∞	∞	0	0	0
Ether-H_2O	90	75	55	0.1	0.1	0.2
Toluene-N NaOH	0.14	0.046	0	1.7	0.1	0
Ether-pH 9	∞	∞	22	0	0	0.1

E_1, estrone; E_2, estradiol-17β; E_3, estriol.

$*$ K = (concentration in upper layer)/(concentration in lower layer).

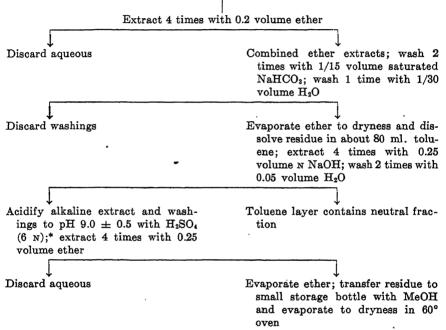† Based on the extraction procedure given in Table II and the formula, $X_n = X_0((K'D)/(K'D + E))^n$, where D is the volume of solvent containing X_0 gm. of solute, E is the volume of the extracting solvent, and X_n is the quantity of solute remaining in the extracted phase after n extractions, and K' is the partition coefficient defined as (concentration in extracted solvent)/(concentration in extracting solvent).

on a Beckman spectrophotometer at 280 mμ. All values obtained by ultraviolet spectrophotometry which differ greatly from unity are approximate and may be in error by as much as a factor of 2, while the error in the more favorable cases may be as high as 25 per cent. The values for the partition coefficients less than 0.05 and greater than 20 are not advantageous for counter-current distribution and hence no effort was made to measure them with great accuracy. They are included in Table III to show the order of magnitude.

No binary two-phase solvent system has yet been found which will give a sufficient spread of the partition coefficients of estrone and estradiol-17β to permit separation by means of a twenty-four transfer counter-current distribution.

For analysis of a counter-current distribution, methods (*a*) and (*c*) (see section on "Methods") are preferred since total fluorescence is obtained directly. Method (*b*) is applicable only when relatively pure compounds

TABLE II

Extraction Procedure

24 hr. urine + 15 volumes per cent 12 N H_2SO_4 (or HCl) autoclaved 20 min. at 20 pounds pressure

|
Extract 4 times with 0.2 volume ether

Discard aqueous   Combined ether extracts; wash 2 times with 1/15 volume saturated $NaHCO_3$; wash 1 time with 1/30 volume H_2O

Discard washings   Evaporate ether to dryness and dissolve residue in about 80 ml. toluene; extract 4 times with 0.25 volume N NaOH; wash 2 times with 0.05 volume H_2O

Acidify alkaline extract and washings to pH 9.0 ± 0.5 with H_2SO_4 (6 N);* extract 4 times with 0.25 volume ether   Toluene layer contains neutral fraction

Discard aqueous   Evaporate ether; transfer residue to small storage bottle with MeOH and evaporate to dryness in 60° oven

* It was found advantageous to add a small amount of concentrated buffer made by mixing 20 volumes of saturated $KHCO_3$ and 1 volume of saturated K_2CO_3. The alkaline solution of estrogens is titrated rapidly to approximately the correct pH by using coarse Hydrion paper. 2 ml. of concentrated buffer per 100 ml. of solution are added, and the pH is adjusted, if necessary, with finely graduated Hydrion paper as indicator.

are distributed. Calculations were made by the method of Williamson and Craig (12).

The result of the distribution of a mixture of pure estrogens is shown in Fig. 3. The components do not overlap at the peaks of the distribution curves. This facilitates the calculation of the partition coefficients and of total amounts of estrogens present. The calculated amounts of estrone,
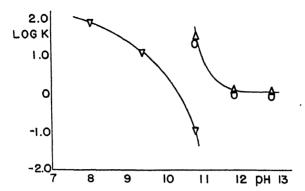
FIG. 2. Effect of pH on the partition coefficients (K) of estrone (◯), estradiol-17β (△), and estriol (▽) in the ether-water system.

TABLE III

*Partition Coefficients of Estrogens in Ternary and Quaternary Solvent Systems**

K = (concentration in upper phase)/(concentration in lower phase).

Upper phase	Lower phase	Estrone	Estra-diol-17β	Estriol
H_2O	CCl_4	0.0	0.08	
10% CH_3OH	"	0.01	0.15	
20% "	"	0.04	0.29	
30% "	"	0.07	0.64	
40% "	"	0.15	1.0	
50% "	"	0.33†	2.1†	16 ± 3†
60% "	"	0.69	4.7	
70% "	"	1.3	7.0	
50% EtOAc-50% C_6H_{12}	H_2O	24‡	7.6	2.9
"	33% C_2H_5OH	39	16†	1.3†
"	50% "	4.2†	2.9†	0.35†
"	70% "	0.73†		0.35†
40% EtOAc-60% C_6H_{12}	40% "	10	5.2†	0.35†
"	50% "	2.6	1.3	0.17
"	60% "	0.78	0.55	0.17
30% EtOAc-70% C_6H_{12}	50% "	2.1	0.92	0.15
10% EtOAc-90% C_6H_{12}	40% "	1.8†	0.65†	0.05†
50% C_2H_5OH	60% $CHCl_3$-40% EtOAc		0.10	0.71
60% C_6H_{14}-40% EtOAc	50% C_2H_5OH	2.2	0.91	0.27
60% CH_3OH	90% $CHCl_3$-10% EtOAc	0.02	0.09	0.50

* All the solvent systems are composed as indicated, by volume. Partition coefficients in other solvent systems may be found in Mather (10) and Bachman and Pettit (13).

† Determined by counter-current distribution.

‡ The numbers in bold-faced type represent measurements obtained on the Beckman spectrophotometer at 280 mμ.

estradiol-17β, and estriol accounted for 99, 96, and 98 per cent of the original estrone, estradiol, and estriol respectively.

When an unknown mixture is distributed, identity of experimental and theoretical curves indicates that a fluorogenically pure component is present. The deviation of the experimental from the theoretical curve is a measure of the amount of impurity. Examination of Figs. 3 and 4 discloses that substantially all of the fluorescence present in the pregnancy urine extracts may be attributed to the three known estrogens. Moreover, the excellent agreement of the partition coefficients (see Figs. 3 and 4 and Table III) indicates strongly that the three compounds are indeed
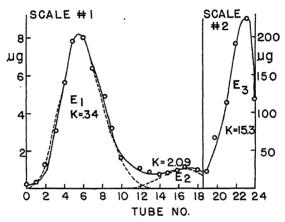
FIG. 3. Counter-current distribution of 45 γ of estrone (E$_1$), 6 γ of estradiol-17β (E$_2$), and 825 γ of estriol (E$_3$). In the upper layer, 50 per cent methanol, 50 per cent water; in the lower layer, carbon tetrachloride. The theoretical and experimental curves for estriol coincide. K is the partition coefficient for each component, calculated from the experimental curve. For all distribution curves, solid line, experimental; dash line, theoretical.

estrone, estradiol-17β, and estriol. Further proof for the identity of the material with $K = 15.4$ was obtained by combining the contents of Tubes 14 to 24 (Fig. 4) and carrying out a second counter-current distribution in a different solvent system. Again agreement between experimental and theoretical distribution curves was obtained (Fig. 5) and the partition coefficient agreed well with the constant previously determined in this solvent system (Table III).

An analysis by counter-current distribution of the phenolic fraction of one-half of a 2 weeks collection of normal male urine was attempted. Daily values ranged from 3 to 21 γ computed as estrone. Estradiol and estriol could not be detected and three distributions failed to separate any

estrone from the overwhelming quantity of a fluorescent material whicl
has a closely similar partition coefficient.

When extracts of urine collected during the pre- and postovulator;
phases of a normal menstrual cycle were distributed, it was found tha·
there was present a large amount of fluorescent material which did no·
correspond in partition coefficient to either estrone, estradiol-17β, or estriol
Nevertheless, in both instances a portion of the curve fitted the theoretica
curve for estrone, thus strongly indicating the·presence of this compound
This was the only known estrogen detected in the pool of preovulator;
phase urine.   However, in the postovulatory phase pool estriol was als(
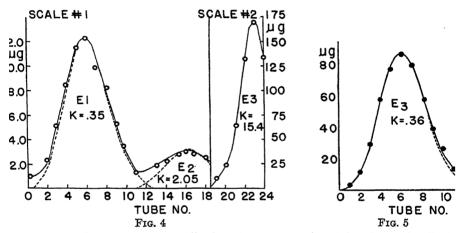
FIG. 4. Counter-current distribution of pregnancy urine of the 9th month.   In th(
upper layer, 50 per cent methanol, 50 per cent water; in the lower layer, carbor
tetrachloride. The theoretical and experimental curves for estriol coincide
E₁, estrone; E₂, estradiol-17β; E₃, estriol.

FIG. 5. Counter-current distribution of the contents of Tubes 14 to 24 of Fig. ·
combined. In the upper layer, 50 per cent ethyl acetate, 50 per cent cyclohexane
in the lower layer, 50 per cent ethanol, 50 per cent water. E₃ is estriol.

present.   Estrone accounted for 9 per cent of the total fluorescence of th(
preovulatory phase pool and estrone and estriol together accounted for 3ξ
per cent of the total fluorescence of the postovulatory phase pool.   Th(
levels of estrogen present in the menstrual cycle represent the smalles·
amounts which can be analyzed by the present technique.

It should be noted that failure to detect a compound such as estradio
in the cycle study does not necessarily mean that the compound is absent
It may be present at too low a level to be detected.   This level depends or
the amount of general, non-specific background fluorescence and on th(
estrogen, but is of the order of 0.01 to 0.05 γ per tube.

It is believed that the non-specific fluorescence of low titer urines may be eliminated, at least in part, by further improvements in the methods of hydrolysis and extraction. Such investigations are now being conducted.

We wish to thank Parke, Davis and Company for their generosity in supplying samples of estrone and estriol and Ciba Pharmaceutical Products, Inc., for estradiol.

We wish also to acknowledge the valuable technical assistance of Miss Katherine Spaulding.

SUMMARY

1. The partition coefficients of the estrogens in a number of solvent systems have been determined.

2. A simplified and efficient extraction procedure based upon these partition coefficients has been designed.

3. By the use of suitable neutral solvent systems in a twenty-four transfer counter-current distribution instrument, the three estrogens may be separated, identified by a physical constant, and quantitatively determined.

4. By this means it has been found (a) that the fluorescence of high titer urines is almost entirely due to estrone, estradiol-17β, and estriol, (b) that there is an overestimation in low titer urines, and (c) that the fluorescence of normal male urine is nearly completely non-specific.

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USE OF BORAX TO LAKE BLOOD FOR OXYGEN DETERMINATION

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Many substitutes for saponin have been proposed for the determination of oxygen and carbon monoxide in blood (1–3), since preparations available in the past few years have not proved satisfactory. Good results for oxygen may be obtained with a reagent containing urea and egg albumin proposed by Hiller, Plazin, and Van Slyke (3), but this reagent requires laborious washing out of the insoluble egg albumin. Since Van Slyke *et al.* (4) found that a borax solution (3 gm. per 100 ml.) can be

TABLE I

Comparison between Results Obtained with Urea and Egg Albumin and with Borax

Experiment No.	Urea-egg albumin			Borax		
	P_1	P_2	Oxygen	P_1	P_2	Oxygen
	mm.	*mm.*	*vol. per cent*	*mm.*	*mm.*	*vol. per cent*
1	169.1	71.6	23.50	168.2	70.9	23.54
2	168.0	71.5	23.05	167.8	71.5	23.01
3	164.3	70.8	22.20	163.7	70.6	22.16
4	158.4	70.3	21.08	158.1	70.1	21.06
5	154.2	69.7	20.28	154.9	70.0	20.34
6	151.4	68.7	20.30	152.4	69.0	20.35
7	150.5	71.0	18.90	150.2	70.9	18.86
8	146.0	70.2	18.13	145.7	70.0	18.08
9	139.1	70.2	16.22	138.5	70.2	16.15
10·	137.4	71.2	15.66	137.8	71.5	15.68

used without saponin for the determination of carbon monoxide in blood, we investigated the possibility of using a similar reagent for the determination of oxygen. In the present note it is shown that oxygen may be determined quantitatively and conveniently with a solution of 6 gm. of borax per 100 ml.

Blood, obtained by venous puncture in more than 100 patients with a variety of clinical conditions, was equilibrated with air and analyzed for oxygen with urea and egg albumin by the method of Hiller *et al.* (3) and with 6 per cent sodium borax ($Na_2B_4O_7 \cdot 10H_2O$) substituted for this reagent. Although it was necessary to keep the borax in solution on cold days by warming the reagent slightly in a pan of water, the reaction

chamber of the apparatus could be readily washed out between determinations.

From the representative results which appear in Table I it is evident that the values obtained with borax are in good quantitative agreement with those determined by the method of Hiller *et al.* (3).

SUMMARY

It is shown that 6 per cent borax may be substituted for saponin or urea and egg albumin to lake blood for oxygen determination. Results obtained with the new reagent agree within 0.5 per cent of those determined with urea and egg albumin.

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THE PREPARATION OF β-DIHYDROEQUILIN*

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In connection with studies in progress in this laboratory on the composition of estrogens in equine pregnancy urine, it became necessary to prepare the heretofore unreported diol, β-dihydroequilin (I). The

reduction of 17-ketosteroids by lithium aluminum hydride leads to the formation of the α-dihydro derivatives exclusively (2), and α-dihydroequilin (II) has been prepared from equilin in this manner.[1] The product was found to be identical with the dihydroequilin first prepared by David (3), using sodium and alcohol as the reducing medium. By the use of the Meerwein-Ponndorf reaction, pairs of isomeric diols have been obtained from estrone and equilenin (4, 5). Therefore, it seemed reasonable to expect that equilin should also produce two 17-dihydro isomers when treated with aluminum isopropoxide.

Haenni[2] has separated α-estradiol from β-estradiol and α-dihydroequilenin from β-dihydroequilenin by partition chromatography. In his procedure dilute sodium hydroxide solution is employed as the immobile solvent adsorbed on Celite and benzene as the mobile solvent. In each case the

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* Although Fieser and Fieser (1) have recently offered evidence that α-estradiol is estradiol-17β (cis), the terms used in this paper to designate steric configuration of the hydroxyl group at C-17 are those that heretofore have been commonly employed: α = trans, β = cis.

[1] Unpublished work of Carol, Haenni, and Banes.

[2] A detailed description of this chromatographic procedure is included in a report on methods of analysis of the estrogenic diols by E. O. Haenni being submitted for publication elsewhere.
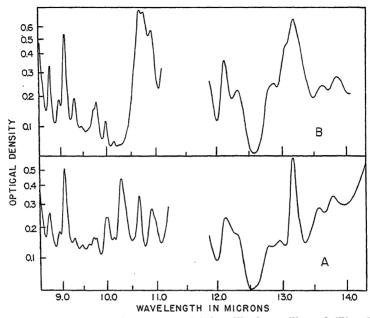
FIG. 1. Infra-red absorption spectra of (A) α-dihydroequilin and (B) β-dihydro-
equilin as their dibenzenesulfonates. Concentration ≈ 4.0 mg. of free diol per ml.
of carbon disulfide. Cell thickness 1.0 mm.
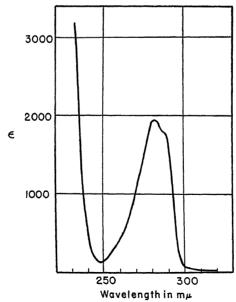
FIG. 2. Ultraviolet absorption spectrum of β-dihydroequilin in alcohol

β isomer exhibits the greater rate of elution and can be obtained in pure form by further treatment of the first portions of eluate received. By applying this partition technique to the products obtained in the Meerwein-Ponndorf reduction of equilin, we have been able to isolate β-dihydroequilin in 20 per cent yield.

The infra-red absorption spectrum of β-dihydroequilin (as the dibenzenesulfonate) (6) is compared with that of α-dihydroequilin (Fig. 1) in the so called finger-print region of 8 to 15 μ. Its ultraviolet absorption spectrum (Fig. 2) has a molecular extinction of 1955 at the 281 mμ maximum, and, as expected, is identical with the spectra of equilin and α-dihydroequilin. The new steroid was found to possess about one-fiftieth of the physiological potency of estrone.[3] Its dibenzenesulfonate melts at 105–106°.

EXPERIMENTAL

β-Dihydroequilin—500 mg. of equilin, m.p. 239°, $[\alpha]_D^{20} = +325°$ (0.5 per cent in alcohol), were heated with 50 ml. of 1.0 M aluminum isopropoxide in isopropanol under a reflux condenser for 5 hours. The isopropanol was gradually distilled until the residual volume was about 10 ml. (3 hours). 25 ml. of cold hydrochloric acid (1:4) were added to the residue and the resultant solution was extracted with two 50 ml. portions of ether. The combined ether extract was washed successively with 10 per cent sodium carbonate solution and water. The ether solution was evaporated to dryness on a steam bath in a current of air. The residue was dissolved in 10 ml. of benzene and chromatographed by the method of Haenni[2] with use of a 25 × 250 mm. column containing 21 gm. of Celite[4] and 20 ml. of 0.4 N sodium hydroxide. After discarding 200 ml. of forerun, 25 ml. fractions of benzene eluent were collected. Those fractions (Nos. 3 to 14) giving a positive test for β-dihydroequilin with a modified Kober reagent[5] were combined and evaporated to dryness on a steam bath in a current of air. The α-dihydroequilin remaining on the column was eluted with about 250 ml. of ether. The β-dihydroequilin residue (containing a small amount of α-dihydroequilin due to overlapping of the bands on the

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[3] We are indebted to Dr. J. M. Curtis and Miss N. M. Hromadka of the Division of Pharmacology, Food and Drug Administration, for carrying out this bioassay.

[4] Celite No. 545, manufactured by Johns-Manville.

[5] 1 part of phenol is dissolved in 1.13 parts by weight of H_2SO_4 without cooling. After 16 to 24 hours there is added to the product 23.5 per cent by weight of diluted H_2SO_4 (1:1.1 by volume). This stable stock reagent is diluted before use with 1.25 volumes of ethanol. When β-dihydroequilin is heated in a boiling water bath with 1 ml. of the diluted reagent for 2 minutes, it develops a blue color with marked red fluorescence. Work is in progress on the development of this reaction for the quantitative determination of β-dihydroequilin.

column) was purified by dissolving in 10 ml. of benzene and rechromatographing on a fresh column as above. Residues from all fractions of eluent (Fractions 6 to 14) yielding material melting at 190° or above were combined and recrystallized from 30 percent alcohol to a constant melting point (two times). Yield 82 mg. of white plates, m.p. 205.5–206°, $[\alpha]_D^{20}$ = +213° (1 per cent in alcohol).

Analysis—$C_{18}H_{22}O_2$. Calculated. C 79.96, H 8.20
                            Found.   " 80.19, " 8.14

β-Dihydroequilin Dibenzenesulfonate—10 mg. of β-dihydroequilin were dissolved in 2 ml. of dry pyridine and mixed with 0.25 ml. of benzenesulfonyl chloride in a glass-stoppered flask. The solution was allowed to stand overnight at room temperature. 25 ml. of water were added to the reaction mixture and the solution was extracted with three 25 ml. portions of chloroform. The combined chloroform extracts were washed with 10 ml. of water and evaporated to dryness on a steam bath in a current of air. The residue was recrystallized from absolute methanol to constant melting point (three times). Yield 12 mg. of white needles, m.p. 105–106°.

Analysis—$C_{30}H_{30}O_6S_2$. Calculated, S 11.64; found, S 11.68, 12.01.

SUMMARY

β-Dihydroequilin was prepared by the Meerwein-Ponndorf reduction of equilin and isolated by means of partition chromatography. The steroid has one-fiftieth the biological potency of estrone.

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ON THE ORIGIN OF THE CARBON CHAIN OF CYSTEINE IN THE RAT*

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Evidence has been presented to show that in rat liver preparations serine is probably condensed with homocysteine to yield cystathionine which is then cleaved to cysteine (1). Employing N^{15}-labeled serine, Stetten found the isotopic nitrogen in the cystine isolated from rat tissues (2). These data served as the basis for the suggestion that the carbons of cystine originate from those of serine.

Sakami demonstrated the synthesis of serine from glycine and "formate" (3) in rat liver, and, later, the same worker showed that serine carbons are synthesized in the rat from glycine and the carbon of the methyl group of choline (4). Prior to that direct evidence was presented to show that the methyl group of sarcosine is oxidized in rat liver preparations to yield formaldehyde (5).

In the present report, data will be described to demonstrate the synthesis of the carbons of cysteine from glycine and the methyl group carbon of methionine in the intact rat.

EXPERIMENTAL

Glycine with the C^{14} in position 2, with the activity of 1.02×10^7 c.p.m. per mg., was obtained from Dr. S. Weinhouse, to whom we wish to express our thanks. Methionine with the C^{14} in the methyl group was synthesized according to Melville *et al.* (6), with the activity of 9×10^4 c.p.m. per mg. Adult female rats of Wistar strain, born and raised in this laboratory, were used. The animals were kept in individual metabolism cages (under a hood) and fed a complete 25 per cent casein diet. Water was allowed *ad libitum*.

1 mg. of radioglycine in water was injected in two equal portions intraperitoneally, 2 hours apart, into each of the two rats. With the second portion of glycine, 100 mg. of bromobenzene were injected, and the urine was collected for the next 48 hours. 40 mg. of radiomethionine in water

were injected in four equal portions intraperitoneally, at 2 hour intervals, into each of the other two rats. With the last portion of radiomethionine, 100 mg. of bromobenzene were injected, and the urine was collected for the next 48 hours. p-Bromophenylmercapturic acid was isolated from the urine by the procedure of McGuinn and Sherwin (7) and recrystallized three times from ethanol with dilute HCl. All isolated samples of mercapturic acid had the correct melting point of 152° (open capillary) and showed no depression when mixed with an authentic sample of ordinary p-bromophenylmercapturic acid. After the activity of the isolated mercapturic acid had been determined, the compound was deacetylated by refluxing in 6 N HCl for 3 hours, and p-bromophenyl-L-cysteine was isolated in the usual manner. The compound melted at 182–183° and showed no depression when mixed with an authentic sample of p-bromophenyl-L-cysteine. The cysteine derivative was then decarboxylated with ninhydrin and the CO_2 was collected as $BaCO_3$.

2 weeks after the injection of the radiomethionine and radioglycine, the hair of all rats was removed by an electric clipper, and pure L-cystine was isolated from the hair hydrolysates (8).

The activity measurements were made directly on the weighed samples of the isolated compounds in a Geiger-Müller counter with a mica window of 1.8 mg. per sq. cm. over an area of 5 sq. cm. Only corrections for background count were made. The quantitative aspect of the activities obtained was not considered in the present work.

Results

The data are summarized in Table I. It is evident that the methyl group carbon of methionine and the carbons of glycine are utilized by the rat for the synthesis of cysteine. On removal of the acetyl group from the mercapturic acid, which was isolated after the administration of radiomethionine to the rat, the specific activity of the carbon of p-bromophenyl-L-cysteine was increased, suggesting that the acetyl group was apparently free of activity. On administration of radioglycine, the specific activity of the mercapturic acid was appreciably greater than the specific activity of p-bromophenyl-L-cysteine isolated after deacetylation, suggesting that the acetyl group in this case was probably radioactive. The problem of possible synthesis of acetic acid from the methyl group of methionine and from glycine is now under investigation. On decarboxylation of the cysteine derivatives, the CO_2 was found free of activity.

The activity of the hair cystine was rather low but real nevertheless. If one considers the fact that the rat hair was not removed prior to the administration of the radioactive compounds and that only 2 weeks elapsed before the hair was removed, the activity of the isolated cystine becomes significant.

Since the present data demonstrate that the methyl group of methionine is available for cysteine synthesis, it is more than likely that the methyl carbon of choline or betaine is similarly utilizable in the rat. Inasmuch as glycine carbons are also participating in the synthesis of the carbon chain of cysteine, it is more than likely that the carbons of the choline and betaine chains are also available for cysteine formation. The latter deductions follow from the observations of Stetten (9) who showed that the nitrogen of betaine finds its way to choline by way of glycine and aminoethanol. Since evidence was presented that choline is probably oxidized to betaine in animals whose liver shows choline oxidase activity (10), glycine can be considered as a metabolic product common to both substances.

TABLE I

Activities of Rat Hair Cystine and of p-Bromophenylmercapturic Acid Isolated from Urine of Rats on Administration of Bromobenzene Together with Either Radioactive Glycine or Methionine Labeled with C^{14} in Methyl Group

Compound administered*	Compound isolated	Activity of carbon
		c.p.m. per mM
Methionine	p-Bromophenylmercapturic acid	300
"	p-Bromophenylcysteine	360
"	Carboxyl carbon of p-bromophenylcysteine	0
"	Hair cystine	24
Glycine	p-Bromophenylmercapturic acid	3824
"	p-Bromophenylcysteine	2892
"	Carboxyl carbon of p-bromophenylcysteine	0
"	Hair cystine	40

* The specific activity of methionine was 2.68×10^6 c.p.m. per mM of carbon and that of glycine was 3.82×10^3 c.p.m. per mM of carbon.

We recently reported the observation that choline augments the utilization of radioisotopic homocystine or homocysteine for the cysteine synthesis in the rat (11). The data reported here offer a possible explanation for this observation, in that the augmentation of the cysteine synthesis by choline from homocystine or homocysteine is due to the utilization of the methyl carbon or of the carbons of the ethanol moiety of choline (or both) for the elaboration of the carbon chain of cysteine via glycine and serine. In experiments now in progress, we are testing the effectiveness of serine and glycine as cysteine precursors from dietary methionine, homocystine, and homocysteine. The interchangeability of serine and glycine in cysteine synthesis from methionine opens interesting possibilities in nutritional studies in which diets are employed with either methionine or homocystine as the sole sulfur amino acid. The results of such studies will be reported at a later date.

SUMMARY

1. Methionine labeled in the methyl carbon with C^{14} and glycine labeled with C^{14} in the amino carbon were administered separately to different rats, together with bromobenzene, and radioactive p-bromophenylmercapturic acid was isolated from the urine. Degradation of the acid revealed the presence of radioactive carbon in the cysteine of the isolated product.

2. Cystine in the hair of the rats which received either radioactive methionine or glycine also was radioactive.

3. The results demonstrate that the methyl group of methionine and the carbons of glycine are utilized by the rat for cysteine synthesis, probably via serine.

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THE PRODUCTS OF OXIDATION OF FATTY ACIDS BY ISOLATED RAT LIVER MITOCHONDRIA*

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Most of the recorded experimental work on the enzymatic oxidation of fatty acids employing *in vitro* methods has been confined to the study of the lower homologues of the long chain acids which predominate in animal tissues and which must be regarded as the physiological substrates of the fatty acid oxidase system. There are two major experimental difficulties in the study of the long chain acids: first, their great insolubility, especially in the presence of Ca^{++} and Mg^{++}, and second, the fact that the fatty acid oxidase system is itself inhibited strongly by excessive concentrations of higher fatty acids (2), an effect which is probably due to their surface activity. It appears likely that these factors were involved in the failures of Muñoz and Leloir (3) and Grafflin and Green (4) to observe oxidation of C_{16} and C_{18} fatty acids by the liver and kidney fatty acid oxidase systems.

Although the use of the short chain acids as model substrates has allowed the accumulation of considerable information in recent years concerning the mechanisms of enzymatic fatty acid oxidation, it must be pointed out that certain gross differences in the metabolism of long and short chain acids are known. It is known, for instance, that administered short chain acids are not stored as such in the tissues or fat depots of the intact animal. Another point of difference is the finding of MacKay *et al.* (5) that administration of short chain acids produces an immediate rise in the blood ketone level of intact, well nourished animals, whereas the long chain acids do not produce this effect. To what extent these gross differences may reflect a fundamental difference in oxidative metabolism of long and short chain acids is not known.

In this paper is recorded a study of the enzymatic oxidation of long

chain acids of the types which predominate in animal tissues as catalyzed by mitochondria isolated from rat liver ("large granules"). When the long chain acids are properly prepared in "solution," they are oxidized by isolated rat liver mitochondria at rates comparable to the rate of oxidation of octanoic acid, a frequently used "model" substrate. A more important finding, however, has been the observation that a much greater proportion of fatty acid carbon is recovered as carbon dioxide than as acetoacetate when long chain acids are oxidized than is the case when the short chain acids are oxidized under identical experimental conditions. Large differences in oxidation products have also been observed when short chain, odd numbered carbon acids were compared with short chain, even numbered carbon acids. Although the data obtained furnish no reason to question the general applicability of the large body of experimental evidence obtained with short chain acids to the oxidative metabolism of the long chain acids, they do raise some questions concerning the metabolic equivalence of the 2-carbon fragments assumed to be formed during the oxidative degradation of fatty acids in the tissues.

<div align="center">EXPERIMENTAL</div>

Methods and Materials—The enzyme preparations employed consisted of suspensions of mitochondria ("large granules") prepared from rat liver by the method of Hogeboom *et al.* (6) as described in a previous communication from this laboratory (7). The suspensions used in the experiments described contained the mitochondria derived from 1 gm. of rat liver suspended in about 4 to 5 ml. of 0.15 M KCl. The general experimental conditions employed were those described previously (7).

The myristic, pentadecanoic, palmitic, and stearic acids used were highly purified products donated by Armour and Company. Oleic, linoleic, and linolenic acids were obtained from the Hormel Institute. They had been purified by bromination-debromination procedures and showed iodine values very close to the theoretical. They were stored under nitrogen to avoid autoxidation. Elaidic acid was prepared from oleic acid with selenium as described by Hilditch and Jasperson (8). Vaccenic acid was a synthetic preparation provided by Professor Frank M. Strong of the University of Wisconsin. Other fatty acids used as substrates were Eastman products. Fatty acids containing more than 12 carbon atoms were tested at a final concentration of 0.00025 M. Octanoate and lower fatty acids were tested at concentrations of 0.001 to 0.002 M. The method of preparing and adding the stock suspensions of the higher fatty acids to the system was found to be of importance in obtaining consistent results. Dilute stock suspensions of the fatty acids in water were warmed to 60° on a water bath, neutralized with the required

amount of NaOH, and the resulting solutions were added while still hot to the Warburg vessels, which contained all other components of the system except the enzyme. The suspension of mitochondria was immediately added and the incubation begun at once. In most of these experiments, a vessel containing octanoate was also included so that results obtained for the higher fatty acids could be compared with those obtained for octanoic acid under identical conditions and with the same enzyme preparation.
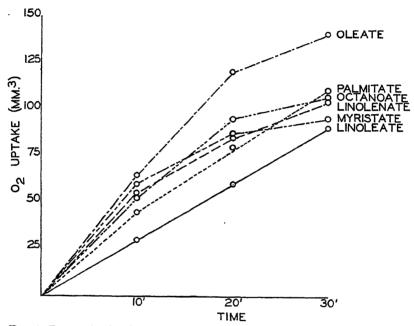

Fig. 1. Rates of oxidation of long chain acids compared to octanoate. Experimental conditions as in Table I.

With the precautions outlined above, it was not difficult to obtain reasonably reproducible oxidation of the higher fatty acids. The rates at which these fatty acids were oxidized, as measured by oxygen uptake, varied somewhat more from preparation to preparation than did the rate at which octanoate was oxidized. This is probably due to the fact that the exact colloidal condition of the substrate, which apparently has an important influence on the enzyme activity, cannot be precisely duplicated. Occasional failures to obtain oxidation of higher fatty acids in suspensions of mitochondria which oxidized octanoate readily can be attributed to this cause.

Rates of Oxidation of Higher Fatty Acids—It was found that both saturated and unsaturated fatty acids containing up to 18 carbon atoms were readily oxidized by rat liver mitochondria. Data illustrating this finding are presented in Fig. 1. These oxygen uptake curves represent *net* uptakes after subtraction of the oxygen uptake due to oxidation of the "priming" or "sparking" Krebs cycle intermediate (3, 9, 10), the addition of which is necessary for oxidation of fatty acids by isolated mitochondria (7). In other experiments, the "unnatural" or trans isomer of oleic acid, elaidic acid, was found to be oxidized quite as well as oleic acid; vaccenic acid, an isomer of oleic acid in which the double bond is in the 11-12
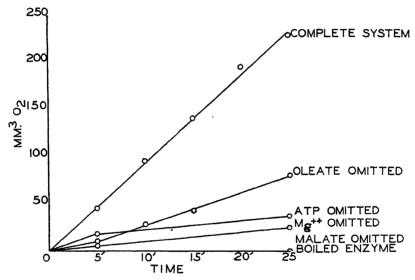
FIG. 2. Cofactor requirements for oxidation of oleate. Conditions as in Table I. When components were omitted, an equal volume of water was substituted.

(trans) position, was likewise rapidly oxidized. Since the unsaturated. fatty acids are known to be autoxidizable, the requirement of cofactors needed for the oxidation of these substances in suspensions of mitochondria was investigated. Fig. 2 shows the results of an experiment in which the cofactor requirements for the oxidation of oleic acid were determined. It can be seen that the same cofactors are required for oxidation of oleate as those which had been shown to be necessary for the oxidation of saturated fatty acids (7, 9). Identical results were obtained when the oxidation of the poly-unsaturated fatty acids, such as linoleic and linolenic acids, was studied. These results indicate that autoxidation processes are probably not involved in the oxygen uptake observed in the

complete system, since autoxidation should have proceeded in the absence of the cofactors mentioned. It may also be concluded that the presence of one or more double bonds in the molecule does not eliminate the necessity for the "priming" oxidation in this system.

Products of Oxidation of Long Chain Acids—The products of fatty acid oxidation in the system employed differed with the chain length of the fatty acid. In Table I are given results of experiments in which the yield of acetoacetate per mole of oxygen utilized and the R. Q. of

TABLE I

Products of Oxidation of Fatty Acids of Varying Chain Length

The Warburg vessels contained final concentrations of 0.001 M adenosine triphosphate, 1×10^{-5} cytochrome c, 0.01 M phosphate buffer at pH 7.4, either 0.0005 M succinate or malate as "priming" substrate, 0.05 M KCl, 0.005 M MgCl$_2$, fatty acids in the concentrations indicated, and 1.0 ml. of mitochondria suspension in a total volume of 3.0 ml. T, 30°; time, between 30 to 45 minutes in the different experiments. Gas phase, air. The values given (in micromoles) have been corrected for endogenous changes due to oxidation of "priming" substrate alone.

Fatty acid	Concentration	O$_2$ uptake	CO$_2$ production	Aceto-acetate production	R. Q.	$\dfrac{\text{Aceto-acetate}}{\text{O}_2}$
	M					
Hexanoic	0.001	9.1	0.5	3.1	0.06	0.34
Octanoic	0.001	7.3	0.9	3.1	0.12	0.42
"	0.001	10.4	0.6	5.9	0.06	0.57
"	0.001	8.9	0.9	4.7	0.10	0.53
Decanoic	0.001	4.5	1.1	2.3	0.24	0.51
Dodecanoic	0.0006	7.3	2.9	1.3	0.40	0.18
Myristic	0.00025	4.3	3.0	0.76	0.70	0.18
Palmitic	0.00025	6.4	3.8	0.70	0.59	0.11
Stearic	0.00025	4.3		0.15		0.04
Oleic	0.00025	6.6	4.5	0.17	0.68	0.03
Elaidic	0.00025	4.2		0.00		0.00
Linoleic	0.00025	4.3		0.23		0.05
Linolenic	0.00025	4.1		0.56		0.14

the oxidation were studied as a function of chain length. Values given are corrected for oxygen uptake, carbon dioxide formation, and acetoacetate production observed in vessels which contained all components of the system except fatty acid. In such control vessels the rate of oxygen uptake was about 15 to 35 per cent of the total uptake of the complete system, the CO$_2$ production was usually that corresponding to an R. Q. of about 1.0 or somewhat less, and there was usually no measurable acetoacetate production. It can be seen that the fatty acids containing 6, 8, and 10 carbon atoms show low R. Q. values and high yields of aceto-

acetate in comparison with members of the series containing 14, 16, and 18 carbon atoms, which showed high R. Q. values and very low production of acetoacetate. The 12-carbon acid represents the transition chain length. Thus with increasing chain length there is a sharp change in the products of oxidation; thus the lower fatty acids yield acetoacetate primarily, while higher members do not give a quantitative yield of acetoacetate but do cause formation of much more carbon dioxide. It appears likely that the Krebs cycle represents the mechanism of CO_2 formation, since other experiments showed that palmitate oxidation in the presence of oxalace-

TABLE II

Comparison of Products of Octanoate and Palmitate Oxidation

Conditions as described in Table I. Time, from 30 to 60 minutes at 30°. Values (in micromoles) have been corrected for endogenous changes. Octanoate and palmitate have been compared with the same mitochondria preparation over the same time interval. Octanoate concentration, 0.001 M; palmitate concentration, 2.5×10^{-4} M.

Experiment No.	Substrate	O_2 uptake	CO_2 output	Acetoacetate formed	R. Q.	$\dfrac{\text{Acetoacetate}}{O_2}$
155	C_8	4.05		1.67		0.41
	C_{16}	3.75		0.15		0.04
192	C_8	9.3		3.52		0.38
	C_{16}	7.7		0.69		0.09
195	C_8	5.7		2.68		0.47
	C_{16}	7.1		0.58		0.08
205	C_8	7.2		3.86		0.54
	C_{16}	5.6		1.00		0.18
208	C_8	7.2		4.03		0.56
	C_{16}	3.6		0.28		0.08
L_7	C_8	7.7	0.7	4.00	0.09	0.52
	C_{16}	5.7	3.2	0.62	0.56	0.11
L_{12}	C_8	8.4	0.8	4.1	0.10	0.49
	C_{16}	6.3	3.7	0.7	0.59	0.11

tate in high concentration caused accumulation of extra citrate. It was also found that when palmitate was added to flasks containing octanoate it did not inhibit acetoacetate formation from the latter, nor did added acetoacetate affect the course of oxidation of octanoate or palmitate.

The difference in oxidation products of the long and short chain acids cannot be explained simply on the basis of a large difference in the rates at which essentially equivalent 2-carbon units are released from short *versus* long chain acids. Three reactions may be listed as determining the over-all pathway of fatty acid carbon in attempts to formulate such an explanation. (1) Rate of oxidative breakdown of the fatty

acid to 2-carbon units. This rate may be (and probably is) substantially different for long *versus* short chain acids. (2) Rate of condensation of the 2-carbon units to form acetoacetate. (3) Rate of oxidation of 2-carbon units to CO_2 via the Krebs cycle.

One possible explanation based on kinetic considerations for the observed differences in oxidation products follows: It is conceivable that the short chain acids may form 2-carbon units at a higher rate than the long chain acids. If the maximum rate of CO_2 formation via the Krebs cycle from these 2-carbon units in the enzyme preparation is greatly exceeded by the rate of formation of 2-carbon units from the short chain acids but is not exceeded by the rate of formation from the long chain acids, then it would be expected that the excess formation of 2-carbon units from the short chain acids might "spill over" and form acetoacetate

TABLE III

Oxidation of Odd Carbon Acids

Experimental conditions as in Tables I and II. Values in micromoles.

Fatty acid	O_2 uptake	CO_2 output	Acetoacetate formed	R. Q.	$\dfrac{\text{Acetoacetate}}{O_2}$
Hexanoic.....................	10.5		3.2		0.31
Heptanoic....................	6.0		0.5		0.08
Octanoic.....................	11.9		4.1		0.34
Nonanoic.....................	9.9		0.5		0.05
Decanoic.....................	12.6		3.9		0.31
Heptanoic....................	7.9	5.0	0.42	0.63	0.05
Nonanoic.....................	12.3	7.1	0.36	0.58	0.03
Pentadecanoic................	4.9	3.8	0.20	0.77	0.04
Heptadecanoic................	3.2	2.1	0.00	0.66	0.00

as a stabilization product, whereas the capacity of the Krebs cycle pathway might be great enough to cause near-by quantitative formation of CO_2 from 2-carbon units derived from the more slowly oxidized long chain acids. Obviously additional explanations based on such kinetic considerations may be constructed. However, it does not appear likely that such considerations can explain the observed differences in view of data presented in Table II, obtained when octanoate and palmitate were studied side by side with identical conditions of time, etc. The data on CO_2 and acetoacetate formation from octanoate *versus* palmitate in Experiments L_7 and L_{12} show conclusively that such a "threshold" hypothesis is untenable. The data do not show, for instance, that acetoacetate is formed from octanoate only when the capacity of the enzyme preparation for CO_2 formation is exceeded (in this case the rate of CO_2 formation from palmitate may be assumed as limiting), nor, conversely, do they show that aceto-

acetate is formed only when the capacity to form CO_2 is exceeded. However, if it is assumed that more than one molecular species of 2-carbon unit is formed from the fatty acid, a more satisfactory explanation can be provided and this will be considered below.

Products of Oxidation of Fatty Acids Containing Odd Number of Carbon Atoms—In the course of this work it was also learned that the odd numbered homologues react in a fashion quite different from the even numbered members in mitochondrial suspensions. Data presented in Table III show the R. Q. values and acetoacetate production observed in the oxidation of odd numbered fatty acids. It will be seen that the short chain *as well as* the long chain acids show preferential oxidation to carbon dioxide, with only very small acetoacetate production. Although these findings are in general agreement with the known behavior of odd numbered carbon acids *in vivo* (see (11)), they differ, at least superficially, from the findings made earlier in this laboratory under somewhat different experimental conditions (12). This will be discussed below.

<center>DISCUSSION</center>

The data presented in this paper clearly show that oxidation of long chain acids by isolated mitochondria may be easily studied if appropriate precautions are made in preparing substrate solutions. Of especial interest is the fact that the unsaturated acids are readily oxidized to CO_2 and that they also require the presence of the "priming" substrate of the Krebs cycle. The lack of specificity of the enzyme system toward the cis and trans isomers of $\Delta^{9, 10}$-stearic acid agrees with the findings of Grafflin and Green on the oxidation of cis and trans isomers of certain lower unsaturated acids (4). It is also noteworthy that the so called essential fatty acids are readily oxidized by isolated mitochondria; these acids are known to be relatively inert metabolically in the intact animal (13, 14).

The observed difference in oxidation products of long and short chain acids requires some comment. The work of Weinhouse et al. (15) and Gurin and Crandall (16) has demonstrated that the oxidation of short chain acids to acetoacetate in both liver slices and washed enzyme preparations similar to those used in this study occurs by successive removal of 2-carbon fragments followed by condensation. Since acetoacetate, once formed, is inert in these liver enzyme preparations (12, 16) and cannot form citrate by condensation with oxalacetate (12), it has been proposed that formation of citrate from fatty acids in these systems occurs by direct condensation of the intermediate 2-carbon fragments with oxalacetate (12). This hypothesis, although not conclusively proved, appears reasonable in view of the large accumulation of data on the relationship of fatty acid and acetoacetate metabolism to the Krebs cycle. There are, there-

fore, two reaction routes for fatty acid oxidation in these liver systems via the intermediary 2-carbon fragments: one proceeding to acetoacetate and the other to CO_2 via the Krebs cycle. The data reported in this paper appear to preclude an explanation for the observed difference in oxidation products based on a disparity in the rates of formation of 2-carbon fragments from the long and short chain acids and the rates of further disposal via the two known pathways. If the 2-carbon intermediate hypothesis is valid, then it would appear as if the 2-carbon fragments derived from long and short chain acids are statistically different, those from the long chain acids being much more reactive with oxalacetate than with each other, and those from the short chain acids being more reactive with each other than with oxalacetate. Recently Crandall, Brady, and Gurin (17) have proved that octanoate may give rise to two different types of 2-carbon fragments. They have measured the ratio of carbon isotope in the carbonyl group to that in the carboxyl group of acetoacetate formed on the oxidation by liver slices of octanoate samples labeled at different carbon atoms. Carboxyl-labeled octanoate formed acetoacetate with an isotope ratio of 0.75 (carbonyl)/(carboxyl), and the same ratio was observed in acetoacetate formed from β-labeled octanoate. However, octanoate labeled in the ζ position yielded acetoacetate with a ratio of 3.3. Their data are consistent with the view that the first three 2-carbon fragments, starting from the carboxyl end of octanoate, are identical and show a somewhat greater tendency to act as acetyl acceptors rather than as acetylating agents in the formation of acetoacetate. However, the terminal 2-carbon unit differs from the rest in that it is far more active in acting as an acetylating agent than as an acetyl acceptor. It is reasonable to assume that these two different species of 2-carbon fragments may likewise differ in their reactivity with oxalacetate to form citrate.

It appears possible to formulate a tentative explanation for the data on long *versus* short chain even numbered carbon acids within the framework of the above considerations. For this purpose it must be assumed that all the 2-carbon units of the fatty acid chain (except the terminal 2-carbon fragment derived from the methyl end) are equal in reactivity, independent of chain length, and that these 2-carbon fragments must have a high reactivity toward oxalacetate, whereas the single unit derived from the methyl end must be assumed to be relatively unreactive toward oxalacetate but very reactive in forming acetoacetate. If these assumptions are true, then it is possible to see how a minimum of approximately 1 mole of acetoacetate could be formed per mole of fatty acid oxidized, regardless of chain length, in the presence of oxalacetate and the Krebs cycle pathway. Under these circumstances CO_2 production from long

chain acids via the Krebs cycle should be considerably greater than from the short chain acids. Calculation of the molar amounts of the 2-carbon units involved in the formation of the CO_2 and acetoacetate, arising from even numbered carbon acids of different chain length, from the data in Tables I and II (assuming 1 μM of 2-carbon units = 2 μM of CO_2 = 0.5 μM of acetoacetate) indicates an approximate fit of the data to this hypothesis, although the deviations are admittedly great. This interpretation of the data is consistent with the evidence of Lorber et al. (18) that the terminal 2-carbon unit of octanoate is less active in forming glycogen than the fragment from the carboxyl end.

It is of interest that O'Connell and Stotz have observed that oxidation of phospholipides by washed liver suspensions proceeded without formation of acetoacetate under the same circumstances in which hexanoate did form acetoacetate (19). They attributed this difference to the fact that in the case of the phospholipide the fatty acids were in esterified form, whereas the hexanoate was present as the free acid anion. It now appears more likely that they observed the effect described in this paper.
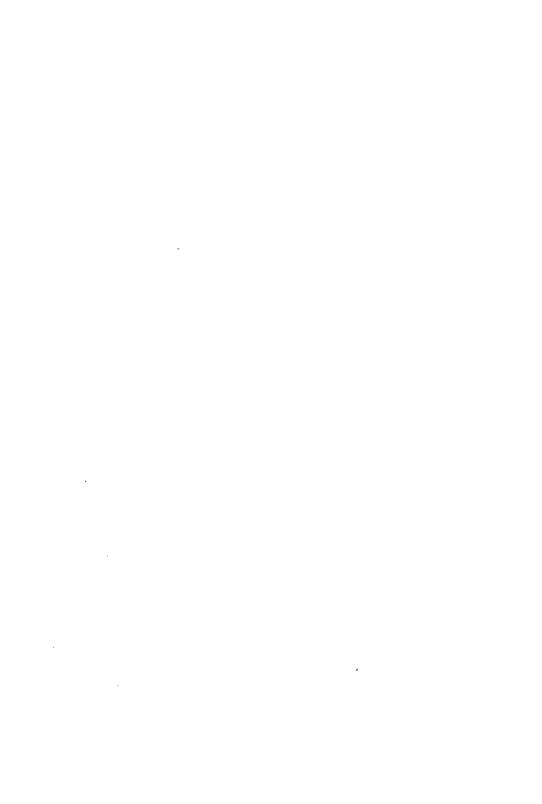
Although the explanation suggested above may appear reasonable for the case of long *versus* short chain, even numbered carbon acids, it falls far short of explaining the differences between the short chain, odd and even numbered carbon acids. The data reported here appear contradictory to previous experiments with short chain, odd numbered carbon acids (12). However, the experiments described in this paper involved the use of a Krebs cycle intermediate as a "primer," whereas the earlier experiments were performed with enzyme preparations which did not require the addition of a "priming" cosubstrate. After the "priming" reaction (3) was "rediscovered" (9, 10), it had been assumed that those enzyme preparations used earlier in this laboratory (2, 12), which did not require addition of a "priming" agent, were active only because they contained trace amounts of Krebs cycle substrates. However, it now appears more likely that the earlier preparations were "primed" or activated by a mechanism other than oxidation of some Krebs cycle intermediate, since they caused formation of large amounts of acetoacetate from odd numbered carbon acids, whereas systems requiring "priming" with a substrate of the Krebs cycle formed only traces of acetoacetate. It is possible that the earlier preparations were already in an "activated" or "primed" state and did not contain sufficient oxalacetate to provide an oxidative pathway other than acetoacetate formation for either odd or even numbered carbon acids. In any event it appears that the nature of the "priming" reaction as well as the existence of two or more species of 2-carbon units may determine to a large extent the balance of oxidation products from the different fatty acids studied.

SUMMARY

Saturated fatty acids of chain length up to 18 carbon atoms, containing both even and odd numbers of carbon atoms, were found to be readily oxidized by mitochondria isolated from rat liver. Oleic, linoleic, and linolenic acids were also readily oxidized, as were the isomeric forms of oleic acid, elaidic acid, and vaccenic acid. The cofactor requirements for the oxidation of the unsaturated fatty acids were found to be identical with those needed for the oxidation of the saturated fatty acids. All fatty acids tested required the presence of a "priming" substrate of the Krebs tricarboxylic acid cycle. It was found that the even numbered fatty acids of chain length greater than 12 carbon atoms gave rise to carbon dioxide as the chief product of oxidation, presumably via the Krebs tricarboxylic acid cycle, in contrast to the shorter fatty acids which yielded primarily acetoacetate under identical experimental conditions. The odd numbered fatty acids, from 7 to 17 carbon atoms in length, were all found to give rise preferentially to carbon dioxide rather than to acetoacetate. The two chief factors involved in these differences in oxidation products appear to be (a) a dissimilarity in metabolic reactivities of the 2-carbon fragments formed in fatty acid oxidation and (b) the nature of the "priming" or "sparking" effect.

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THE FAILURE OF ACETYLDEHYDROTRYPTOPHAN TO SUPPORT THE GROWTH OF THE RAT*

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A study of the metabolism of acetyldehydroamino acids was suggested by the observations that the acetyldehydroamino acid can be readily reduced *in vitro* to the corresponding acetylamino acid and can be hydrolyzed to the related keto acid. It was known from the work of other investigators that either the acetyl-L derivatives or the keto acid analogues of the essential amino acids will support the growth of young rats in lieu of the amino acid. This applied to all the acetyl-L derivatives except α-N-acetyl-L-lysine (1) and all the keto acids with the exception of α-keto-β-hydroxybutyric acid which appears not to have been studied (2–5).

The acetyldehydroamino acids hold particular interest in that they possess the carbon chain which is recognized as being the principal part of the essential moiety of the amino acid, and they contain the nitrogen needed by the organism. The compounds, having no center of asymmetry, are not optically active; so that the question of the utilization of optical isomers does not arise.

In order to test the possibility of the utilization of acetyldehydro compounds, acetyldehydrotryptophan was synthesized for feeding experiments. The general procedure of Bergmann and Stern (6) was applied. Chloroacetyl-DL-tryptophan was converted to an oxazolone by heat and acetic anhydride. The free acid was obtained by the alkaline hydrolysis of the oxazolone. It was characterized by conversion to known derivatives. When fed to young rats at the level of molar and 2 molar equivalents to DL-tryptophan, acetyldehydrotryptophan did not support growth. The animals likewise failed to gain weight when acetyldehydrotryptophan was supplied by subcutaneous injection.

The acetyldehydroamino acid was tested for an antagonism to tryptophan. The amount of DL-tryptophan in the diet was lowered experimentally until a level was attained which would just maintain the weight of the animals. The addition of acetyldehydrotryptophan to this ration did not produce a loss of weight.

*Chloroacetyl-*DL-*tryptophan*—The compound was prepared by the method of Abderhalden and Kempe (7). After recrystallization from water and ethanol, the product melted at 154–155°.

Acetyldehydrotryptophan—2.5 gm. of chloroacetyl-DL-tryptophan were heated at 100° for 1 hour with 30 ml. of redistilled acetic anhydride. The solution was concentrated *in vacuo* at 50° to remove the excess acetic anhydride. A mixture of 10 ml. of methanol and 10 ml. of dioxane was used to dissolve the residue. 5 ml. of 2 N sodium hydroxide, an amount sufficient to make the solution alkaline to phenolphthalein, were added to the solution and the solution was allowed to stand overnight at 7°. Organic solvents were removed by concentration to half volume at 50° *in vacuo*. The resulting solution was poured over 400 ml. of cracked ice and water and was made acid to Congo red with 3 ml. of concentrated hydrochloric acid. A reddish brown precipitate separated. After standing at room temperature about 30 minutes, the solution was filtered. The product, dried in air, was 2.25 gm. of reddish brown crystalline material. After several recrystallizations from methanol the melting point of the crystals was constant at 185–186°. The maxima in the absorption spectrum were 8160 at 280 mμ and 9100 at 335 mμ. Nitrogen was determined by the Dumas method.

$C_{13}H_{12}O_3N_2$.   Calculated.   N 11.4, neutral equivalent 244.2
(244.2)   Found.   " 11.3   "   "   244

200 mg. of acetyldehydrotryptophan were dissolved in 10 ml. of 40 per cent sodium hydroxide. After heating on a boiling water bath for 6 hours, the solution was poured into 90 ml. of water and made acid to Congo red with 5 N sulfuric acid. Ether was used to extract the indolepyruvic acid. The ether was removed by concentration at room temperature *in vacuo*, and the residue dissolved with 5 ml. of ethanol. The *p*-nitrophenylhydrazone of indolepyruvic acid was prepared from this solution by the procedure of Ellinger and Matsuoka (8). The melting point of a sample which had been recrystallized twice from benzene was 154°, the value reported in the literature.

200 mg. of acetyldehydrotryptophan were dissolved in 20 ml. of a solution half saturated with sodium carbonate. About 100 mg. of Raney's nickel catalyst (9) were added. The progress of the reduction was followed by the decrease in the maximum of absorption at 335 mμ. When the maximum had disappeared, the solution was filtered and acidified. A brown precipitate, m.p. 199°, was obtained. On standing, the solution

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[1] All melting points are corrected.

deposited a second crop of buff crystals which melted at 203°. The product was shown to be identical with an authentic sample of acetyl-DL-tryptophan by a determination of the melting point of a mixture of the two.

5 mg. of the crystals obtained from the reduction of the acetyldehydrotryptophan were dissolved in 1 ml. of acetone and 1 drop of morpholine was added. An oil which separated from the solution was crystallized by treatment with methanol and ethyl acetate. The melting point of these crystals and of a sample of the morpholine salt prepared from pure N-acetyl-DL-tryptophan was 177°, and the melting point of a mixture of the two samples was the same.

Acetyl-DL-tryptophan was prepared by the method described by du Vigneaud and Sealock (10). It melted at 204°.

Feeding Experiments

Young rats of the Yale strain were used. Six litter mates, each weighing about 50 gm., were placed in individual cages and supplied with food and water *ad libitum*. DL-Tryptophan, or its derivatives, and B vitamins were added to the diet at the time of the placing of the food in the cages. The composition of the diets was amino acid mixture[2] 20, sucrose 32, cornstarch 32.9, salt mixture, Jones-Foster (11), 4, corn oil 2, lard 7, Cellu flour 2, cod liver oil 0.05, inositol 0.01, choline chloride 0.02, α-tocopherol 0.002 per cent. The following vitamin supplement was used per kilo of diet: thiamine hydrochloride 5 mg., riboflavin 10 mg., pyridoxine hydrochloride 5 mg., nicotinic acid 5 mg., calcium d-pantothenate 25 mg., p-aminobenzoic acid 300 mg., 2-methyl-1,4-naphthoquinone 2 mg. Growth measured as a change in weight is shown in Table I.

When the animals failed to gain weight on acetyldehydrotryptophan, the possibility that the material was not being absorbed was investigated. In addition to the compound administered in the diet, 12 mg. were injected daily in three subcutaneous doses. For injection the compound was dissolved in sodium bicarbonate and the pH was adjusted to 7.4 by equilibration with carbon dioxide gas. The animals on this regimen continued to lose weight and died in about 8 days.

In order to test whether or not acetyldehydrotryptophan was toxic or antagonistic to tryptophan, a second series of feeding experiments was performed with four animals. The amount of tryptophan added to the ration was lowered until it was found that a 0.02 per cent DL-tryptophan

[2] Amino acids, lyophilized (Interchemical Corporation, Union, New Jersey), a specially prepared mixture of amino acids produced by complete acid hydrolysis of casein, modified by partial removal of aspartic and glutamic acids, and not supplemented with tryptophan.

level would just maintain the weight of the animals.   When 0.4 per cent acetyldehydrotryptophan was added to this diet, no loss of weight was observed over a period of 8 days.

The excretion of tryptophan, or substances reacting to the Hopkins-Cole test, was studied in order to trace further the fate of the acetyldehydro derivative. · An adult male rat, weight 150 gm., was given 100 mg. of DL-tryptophan in 50 gm. of the basal diet.   The urine samples were col-

TABLE I

Growth Response to DL-Tryptophan and Its Derivatives

Rat No.	Initial weight	No. of days	Total food eaten	Change	Supplement
	gm.		*gm.*	*gm. per day*	
23	60	20	144	+2.0	0.2% DL-tryptophan
		4	26	−1.9	None
		4	18	−1.6	0.4% acetyldehydrotryptophan
		7	27	−1.8	0.4% " and 12 mg. injected per day
24	63	28	219	+1.9	0.2% DL-tryptophan
		8	43	−1.5	None
25	65	4	21	+0.6	0.2% DL-tryptophan
		8	44	−0.9	None
		8	50	+1.2	0.2% chloroacetyl-DL-tryptophan
		16	118	+1.7	0.4% "
26	74	4	23	+0.6	0.2% DL-tryptophan
		8	47	−1.8	None
		24	160	+1.5	0.2% chloroacetyl-DL-tryptophan
27	63	16	100	+2.4	0.2% DL-tryptophan
		8	43	−0.8	None
		8	39	−0.3	0.2% acetyldehydrotryptophan
		4	15	−0.6	0.4% "
28	74	4	18	+1.2	0.2% DL-tryptophan
		8	45	−1.4	None
		8	39	−0.6	0.2% Acetyldehydrotryptophan
		8	33	−0.7	0.4% "
		6	38	−1.0	0.4% " and 12 mg. injected per day

lected under toluene during the 4 days required for the diet to be eaten. After a 2 day rest, the animal was given 50 gm. of diet containing acetyl-dehydrotryptophan equivalent to the tryptophan used in the first feeding. The urine was collected for 5 days while the diet was being eaten.   Quantitative Hopkins-Cole tests (12) on the two urine samples were positive. Significantly greater amounts of material responding to the Hopkins-Cole test were found in the urines after the feeding of acetyldehydrotryptophan than after the amino acid.

DISCUSSION

Growth-supporting experiments of the type described in this paper have long served as a screening test for the over-all ability of the animal body to carry out chemical transformation of an administered derivative into an essential metabolite. The limitations of the method may be gaged by the fact that the body contains systems capable of converting D-amino acids to utilizable keto acids; yet several of these D-amino acids will not replace the corresponding L isomer in the diet. The failure of the animals to grow when acetyldehydrotryptophan replaced DL-tryptophan in the diet indicates only that the compound is not utilizable for growth per se nor is it metabolized to the keto acid or to the acetylamino acid at a rate sufficient to support growth. Thus the compound is not significantly affected by the hydrolytic activity of the digestive processes or the reducing action of the intestinal bacteria.

The data show that the diet, when supplemented with DL-tryptophan or chloroacetyl-DL-tryptophan, will support growth. Other investigators have shown that both acetyltryptophan (13) and indolepyruvic acid (14) will support the growth of rats when fed with similar diets. "Good growth" such as is obtained with liver powder and other supplements was not attempted in this study. The question of absorption of the acetyldehydrotryptophan was met by subcutaneous injection of the compound, an administrative route which is adequate for acetyltryptophan (15).

These findings for the integrated enzyme systems of the intact animal are in harmony with the results of Price and Greenstein (16) who have shown that with the isolated dehydropeptidase systems most acetyldehydroamino acids do not meet the requirements for a substrate.

The failure of the animals to lose weight when acetyldehydrotryptophan was administered on a low tryptophan diet suggests that the compound is not a metabolic antagonist to tryptophan in the rat.

SUMMARY

Acetyldehydrotryptophan has been prepared by heating the chloroacetyl-DL derivative with acetic anhydride and hydrolyzing the resulting oxazolone to the free acid. The compound was characterized by reduction to N-acetyl-DL-tryptophan and by hydrolysis to indolepyruvic acid.

Young rats were fed a tryptophan-deficient diet. Protein was substituted by a mixture of amino acids, prepared by complete hydrolysis of casein with acid, followed by removal of part of the dicarboxylic acids. When acetyldehydrotryptophan was added to this diet at molar and 2 molar equivalent weights to 0.2 per cent DL-tryptophan, it did not support growth of young rats. The same amount of DL-tryptophan or chloroacetyl-DL-tryptophan fed with the same diet produced satisfactory growth.

Acetyldehydrotryptophan did not produce a weight loss when fed with a diet containing minimum amounts of tryptophan.

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A STUDY OF NON-COMPETITIVE ANTAGONISM WITH CHLOROMYCETIN AND RELATED ANALOGUES OF PHENYLALANINE

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When the structure of Chloromycetin became known (1), the resemblance of this useful antibiotic agent to several important metabolites could be perceived. Either phenylalanine or tyrosine could be visualized as being converted into the drug by carrying out four structural alterations, three of which were each known, from work with other antimetabolites (2), to be capable of transforming an essential metabolite into an antagonist of it. The relationship of Chloromycetin to phenylalanine can be seen in Fig. 1. β-Hydroxyphenylalanine was already known to be a competitive antagonist of phenylalanine (3), and alcohols such as pantothenol derived from acidic vitamins by replacement of the —COOH group with —CH_2OH had been shown to function similarly (4). The working hypothesis therefore arose that Chloromycetin owes its antibacterial properties to an ability to call forth in the organisms a deficiency of an essential metabolite. The problem was to identify the metabolite, because the drug resembled several, and to determine whether this metabolite would antagonize the action of the antibiotic agent.

Experiments are described in which the toxicity of Chloromycetin for two species of bacteria was found to be decreased by phenylalanine. In one of these, tyrosine and tryptophan were also shown to exhibit such an antagonism, but reasons for regarding their action as being due to metabolic involvement with phenylalanine will be presented.
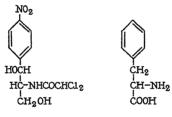
Although phenylalanine was able to overcome the toxicity of minimally effective doses of Chloromycetin, the antagonism was not competitive, and, indeed, could not be shown with large amounts of the drug. An effort was therefore made to determine whether competitive inhibitors of bacterial growth could be made by introduction into phenylalanine of some, but not all, of the four alterations relating Chloromycetin to it. It was found that analogues possessing less than four of these modifications did check bacterial growth in competition with phenylalanine, but that the introduction of substituents such as the nitro and dichloroacetyl groups contributed to a type of toxicity which could not be overcome

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* With the technical assistance of N. Smith and E. A. Singer.

with the metabolite. Because these observations have a bearing on the understanding of the basic mode of action of antimetabolites, and on the interpretation of non-competitive antagonism between harmful agents and essential metabolites which vaguely resemble them, they are presented in this paper.

<div align="center">EXPERIMENTAL</div>

Sources and Preparation of Substances—Chloromycetin, DL-*threo*-1-phenyl-2-amino-1,3-propanediol, D-(−)-*threo*-1-(*p*-nitrophenyl)-2-amino-1, 3-propanediol, and DL-*threo*-1-phenyl-2-dichloroacetamido-1,3-propanediol were kindly supplied by Dr. L. A. Sweet of Parke, Davis and Company (5). β-Hydroxyphenylalanine was prepared from benzaldehyde and glycine according to the directions of Erlenmeyer and Früstück (6). Its spatial configuration was unknown. *N*-Dichloroacetylphenylalanine was

FIG. 1. Structures of Chloromycetin and phenylalanine

made by acylation of the amino acid with dichloroacetyl chloride in cold aqueous alkali in the usual fashion. When recrystallized from water, it melted at 140–142° and contained 5.2 per cent of nitrogen.

DL-*1-Hydroxy-2-amino-3-phenylpropane*—An ether solution of 9.5 gm. of DL-phenylalanine ethyl ester was slowly added to 1.1 gm. of lithium aluminum hydride dissolved in 100 cc. of anhydrous ether. The mixture was stirred for 20 minutes, and then treated with 100 gm. of ice and 8 cc. of concentrated hydrochloric acid. After a few minutes, the aqueous phase was separated, made strongly alkaline with NaOH, and heated on the steam bath for an hour in order to hydrolyze any remaining ester. The alkaline solution was then extracted continuously with ether for 3 hours. The extracted liquid product (4.1 gm.) was crystallized as the hydrochloride by passing dry HCl into a dry ether solution of it. In order to remove any trace of phenylalanine ester which might have remained, the hydrochloride was dissolved in 10 cc. of 4 N NaOH, and, after the solution had stood for some time, the base was again obtained by continuous extraction with ether, and the hydrochloride was prepared as

before and recrystallized from alcoholic solution by addition of ether. The crystals softened at 145° and melted at 159°. This, as well as other melting points, was determined on a hot stage microscope. Adkins and Pavlic (7) reported a melting point of 156° for the same compound prepared by catalytic reduction of phenylalanine ethyl ester.

$C_9H_{14}ONCl$. Calculated, N 7.5; found, N 7.6

N-Dichloroacetyl-β-hydroxyphenylalanine—This substance was prepared by acylation of 9 gm. of β-hydroxyphenylalanine in cold (−10°), aqueous NaOH with 7 gm. of dichloroacetyl chloride. The product was precipitated by addition of HCl and was recrystallized from water. M.p. 164°; yield, 10.6 gm.

$C_{11}H_{11}O_4NCl_2$. Calculated, N 4.8, Cl 24.4; found, N 5.0, Cl 23.8

During the acylation, the odor of benzaldehyde became plainly discernible. Each time the product was recrystallized from water this odor was again detected. However, the β-hydroxyphenylalanine did not smell of benzaldehyde.

In order to determine whether dehydration to the corresponding dehydrophenylalanine derivative had occurred, the ultraviolet absorption spectrum was examined. No maxima were found in the region in which acetyldehydrophenylalanine shows good absorption. Furthermore, the fact that benzoylation of β-hydroxyphenylalanine led to a benzoyl derivative quite different in melting point (156°) from benzoyldehydrophenylalanine (225°) likewise showed that dehydration of such acyl derivatives of β-hydroxyphenylalanine could be avoided.

N-Acetyl-β-hydroxy-p-nitrophenylalanine—18 gm. of β-hydroxyphenylalanine were acylated with 10 gm. of acetyl chloride in a manner similar to that just described. Acidification of the reaction mixture resulted in no precipitation, and therefore the product was extracted with ethyl acetate and was obtained as 14.5 gm. of crystalline solid. Without further purification this was dissolved in 25 cc. of cold (−14°), fuming nitric acid. The compound was added slowly to the acid to avoid excessive production of heat. The solution was then poured on 80 gm. of ice, and the crystals, which separated slowly, were collected and recrystallized from water. Yield, 3.3 gm.; m.p. 186–187°. The melting point depended somewhat on the rate of heating. A further 7.5 gm. were obtained by extraction of the mother liquors at pH 3 with ethyl acetate.

$C_{11}H_{12}O_6N_2$. Calculated, N 10.4; found, N 10.1

β-Hydroxy-p-nitrophenylalanine—2.0 gm. of N-acetyl-β-hydroxy-p-nitrophenylalanine were suspended in 25 cc. of 6 N HCl, and the mixture was refluxed overnight. Excess acid was removed by repeated evaporation

under reduced pressure. Unchanged starting material and colored decomposition products were extracted from the aqueous solution with ethyl acetate. The desired compound was finally obtained by solution of the residue in alcohol and precipitation by neutralization with diethylamine. The material so obtained was purified by recrystallization from dilute alcohol. On a paper strip chromatogram (8) with phenol saturated with 0.1 N HCl as solvent, it showed a single spot with R_F 0.68. Yield, 1.2 gm.

$C_9H_{10}O_5N_2$. Calculated, N 12.4; found, N 12.2

Substance with High Antibacterial Activity from Reaction of Glycine with p-Nitrobenzaldehyde—An attempt was made to prepare β-hydroxy-p-nitrophenylalanine by condensation of glycine with p-nitrobenzaldehyde under the conditions which, with benzaldehyde itself, yielded β-hydroxyphenylalanine (6). A very vigorous reaction occurred with evolution of ammonia and production of a dark red color. After decomposition of the Schiff base with acid, the product was found to be markedly low in nitrogen. By extraction with alcoholic HCl and neutralization of the extract with diethylamine, a small amount of the substance was isolated which was still quite impure, but which had the properties of an amino acid. It migrated on a paper strip chromatogram with phenol saturated with 0.1 N HCl, and showed as a single ninhydrin-reactive spot with R_F 0.08. Although a pure substance could not be isolated, the most active concentrate, which was obtained in minute yield, caused half maximal inhibition of the growth of *Escherichia coli*, under the conditions to be described shortly, when 2 γ of it per cc. of culture medium were used. From its high activity and slow rate of migration on a paper strip chromatogram, the conclusion was obvious that it was not identical with the β-hydroxy-p-nitrophenylalanine just described.

N-Dichloroacetyl-β-hydroxy-p-nitrophenylalanine—Acylation of 1.8 gm. of β-hydroxy-p-nitrophenylalanine with 1.5 gm. of dichloroacetyl chloride in the presence of cold, aqueous NaOH led to the isolation of N-dichloroacetyl-β-hydroxy-p-nitrophenylalanine. After recrystallization from water, this compound melted at 158–162°.

$C_{11}H_{10}O_6N_2Cl_2$. Calculated, N 8.3; found, N 8.2

Two attempts to prepare this substance by direct nitration of N-dichloroacetyl-β-hydroxyphenylalanine led only to amorphous materials which could not be induced to crystallize. One of these had an antibacterial potency about half that of the crystalline substance.

N-Dichloroacetyl-p-nitrophenylalanine—To 2 cc. of fuming nitric acid cooled in an ice-salt bath, 2 gm. of dichloroacetylphenylalanine were added

in small portions. The solution which resulted was held in the cold for a few minutes, and was then poured on 25 gm. of ice. The product crystallized readily, and was recrystallized from water. The yield was 1.5 gm. of crystals which melted at 177–179°.

$$C_{11}H_{10}O_5N_2Cl_2. \quad \text{Calculated, N 8.7; found, N 8.7}$$

When this substance was hydrolyzed with boiling 6 N HCl, it yielded *p*-nitrophenylalanine, a compound previously described in the literature.

Configuration of Derivatives of β-Hydroxyphenylalanine—Since only one of the four possible isomers of Chloromycetin exhibits antibacterial properties (5) any interpretation of the findings with derivatives of β-hydroxyphenylalanine must take into account their configuration about the two asymmetric carbon atoms. The derivatives of 1-phenyl-2-amino-1,3-propanediol which were supplied by Parke, Davis and Company were of the *threo* series, the same as that of Chloromycetin. Presumably the β-hydroxyphenylalanine herein described was a mixture of diastereomers, since it was formed from two optically inert substances. Therefore its acyl and nitro derivatives should have been mixtures, unless complete separation of diastereomers had occurred readily during purification. Attempts were made to establish whether these substances belong to the *erythro* or the *threo* series or whether they were mixtures. Likewise, efforts were made to oxidize Chloromycetin to *N*-dichloroacetyl-β-hydroxy-*p*-nitrophenylalanine for comparison with the synthetic material. However, these attempts were unsuccessful and no clear evidence for the configuration of the compounds herein described was obtained. If these materials were diastereomeric mixtures, the values for their antibacterial potencies would be only of qualitative importance. However, if one racemate were inactive, as is true with Chloromycetin, any reversible toxicity which the synthetic substances might show would be interpretable even though their configurational purity was unknown.

Organisms—*Lactobacillus casei* and *E. coli* were the strains which have been used over the past decade in this laboratory. The phenylalanine-requiring mutant of *E. coli* was one kindly supplied by Dr. B. Davis. He had found it to respond only to phenylalanine and to phenylpyruvic acid, and not to be capable of using tyrosine or tryptophan in place of these metabolites.

Non-Competitive Antagonism between Chloromycetin and Phenylalanine in Growth of E. coli—When *E. coli* was grown in the synthetic medium of Hook *et al.* (9), the inhibitory effects of small doses of Chloromycetin were overcome completely by addition of DL-phenylalanine to the medium. As the concentration of the drug was increased, the antagonism with the metabolite could no longer be demonstrated. Data to illustrate this

point are shown in Table I, where it can be seen that, while the toxic effects of 1 γ of Chloromycetin per cc. could be nullified by sufficient phenylalanine, the growth-inhibitory properties of 2 γ of the drug were scarcely influenced by the amino acid. For these experiments 10 cc. of the basal medium, composed of glucose, ammonium chloride, sodium chloride, potassium and sodium phosphates, and magnesium sulfate, were used in each tube. The contents were sterilized by autoclaving, cooled, and inoculated with 0.05 cc. of a 20 hour culture of the organism, diluted 1:50. The inoculum was grown in Todd-Hewitt broth (10). After the seeding, all tubes were incubated at 37° for 18 to 20 hours, and then the turbidity of

TABLE I

Antagonism between Chloromycetin and Phenylalanine in Growth of E. coli in Synthetic Medium

Chloromycetin	DL-Phenylalanine	Transmittance*
γ *per cc.*	γ *per cc.*	
0	0	67
0.5	0	67
1.0	0	83
2.0	0	98
5.0	0	98
0	500	67
1.0	500	67
1.0	200	75
1.0	100	76
1.0	50	83
2.0	500	95
2.0	200	96

* Expressed as per cent of incident light transmitted in comparison to the uninoculated basal medium.

the contents of each was measured in an Evelyn photometer. The composition of the basal medium appeared to be of importance to the demonstration. Thus, when the nitrogen source was a mixture of pure amino acids, such as in the medium of Hac *et al.* (11), no antagonism of the action of Chloromycetin with phenylalanine could be observed. However, the effect which has been described in the simplified medium of Hook *et al.* (9) was not ascribable to a non-specific betterment of the nitrogen nutrition of the organisms, because other amino acids were ineffectual and growth in the absence of the drug was not stimulated by phenylalanine (see Table I).

Effects of Other Amino Acids on Toxicity of Chloromycetin for E. coli— Other amino acids were tested singly for ability to overcome the inhibition

of growth of *E. coli* caused by Chloromycetin. For most of these tests, an amount of the drug just sufficient to cause half maximal failure of growth under the conditions defined in the previous section was used. In each experiment the effect of phenylalanine was simultaneously determined in order that a positive control might always be available for comparison. For those amino acids which proved active, separate trials were made of their ability to overcome twice this amount of Chloromycetin, and 10 times as much of it. None was found to be better in this respect than was phenylalanine. Of the amino acids tested, L-tyrosine and DL-tryptophan showed some ability to overcome the action of Chloromycetin, while glycine, alanine, threonine, leucine, isoleucine, valine, aspartic acid, glutamic acid, arginine, and lysine were without effect. Each amino acid was examined in amounts up to 0.5 mg. per cc. of culture medium. Approximately the same amount of tyrosine or of tryptophan as of phenylalanine was needed to achieve comparable activity. Results similar to

TABLE II

Toxicity of Chloromycetin for L. casei in Presence of Increased Concentrations of Tyrosine or Phenylalanine

L-Tyrosine	DL-Phenylalanine	Chloromycetin allowing half maximal growth
γ *per cc.*	γ *per cc.*	γ *per cc.*
20	20	1.2
500	20	1.2
20	500	1.8

these were found when the phenylalanine-requiring strain of *E. coli* was used instead of the wild type.

Failure of Tyrosine to Antagonize Action of Chloromycetin on L. casei— The activity of tyrosine, and possibly also of tryptophan, was concluded to arise from the ready conversion of these to phenylalanine. Such interrelationships have been implied in several recent studies with *E. coli* (see for example Beerstecher and Shive (12)). Experimental evidence compatible with this view was collected by use of an organism which required both tyrosine and phenylalanine in the medium and therefore was unable to make one from the other. Such an organism is *L. casei*. For these experiments, the amino acid-containing medium of Hac *et al.* (11) was employed, except that just sufficient tyrosine and phenylalanine to meet the demands for optimal growth were included (20 γ per cc.). The amount of Chloromycetin which would cause half maximal inhibition of growth was then determined. The same estimations were then made in the presence of excess tyrosine and of excess phenylalanine. The tech-

TABLE III

Toxicity for Phenylalanine-Requiring Strain of E. coli of Compounds Intermediate in Structure between Chloromycetin and Phenylalanine, and Ability of Phenylalanine to Overcome It

Compound No.	Name	Toxicity*				Type of antagonism
		With 5 γ phenylalanine	With 10 γ phenylalanine	With 20 γ phenylalanine	With 200 γ phenylalanine	
1	Dichloroacetylphenylalanine	Phenylalanine activity				
2	1-Hydroxy-2-amino-3-phenylpropane	Nothing at 4.0				
3	p-Nitrophenylalanine	Nothing at 1.0	Nothing at 1.0			
4	β-Hydroxyphenylalanine	1.0, never complete	6.8	13.0		Competitive†
5	1-Hydroxy-2-dichloroacetamido-3-phenylpropane	Nothing at 0.6				
6	β-Hydroxy-p-nitrophenylalanine	0.8		1.2	Nothing at 2.0	Non-competitive
7	Dichloroacetyl-p-nitrophenylalanine	6.0	8.0			Non-competitive
8	1-Phenyl-2-amino-1,3-propanediol	Nothing at 2.0		Nothing at 6.0		
9	N-Dichloroacetyl-β-hydroxyphenylalanine	Nothing at 3.0				
10	1-Phenyl-2-dichloroacetamido-1,3-propanediol	0.05	0.10	0.20	0.10	Competitive below 0.2 mg., then irreversible
11	N-Dichloroacetyl-β-hydroxy-p-nitrophenylalanine	0.5	1.0	1.5		Competitive below 1.5 mg.
12	1-(p-Nitrophenyl)-2-amino-1,3-propanediol	0.2		0.6	0.6	Competitive below 0.6 mg., then irreversible
13	Chloromycetin	0.0018	0.0027		0.0031	Non-competitive

* Expressed as the amount (in mg. per cc. of culture) required to cause half maximal inhibition of growth in the presence of graded concentrations of phenylalanine.

† The competitive nature of the antagonism was obscured in the presence of minimal concentrations of phenylalanine, probably because of some apparent metabolite activity of the analogue under these conditions (see Fig. 2).

nique of inoculation was the same as that described for the trials with *E.
coli*. Incubation was at 37° for 40 hours. Under these conditions, only
phenylalanine, and not tyrosine, was the amino acid capable of antago-
nizing the toxicity of the drug (see Table II). Therefore, it was con-

TABLE IV

*Toxicity for Wild Strain of E. coli of Compounds Intermediate in Structure between
Chloromycetin and Phenylalanine and Ability of Phenylalanine
to Overcome It*

Compound No.	Name	Toxicity*				Type of antagonism
		With 0 γ phenyl-alanine	With 10 γ phenyl-alanine	With 20 γ phenyl-alanine	With 200 γ phenyl-alanine	
1	Dichloroacetylphenyl-alanine	Nothing at 3.0				
2	1-Hydroxy-2-amino-3-phenylpropane	2.0		3.0	3.0	Non-competitive and slight
3	*p*-Nitrophenylalanine	0.6			0.75	" "
4	β-Hydroxyphenylalanine	0.3	4.8	10.0		Competitive
6	β-Hydroxy-*p*-nitrophenyl-alanine	0.8		1.2	1.2	Non-competitive
7	Dichloroacetyl-*p*-nitro-phenylalanine	Nothing at 3.0				
8	1-Phenyl-2-amino-1,3-propanediol	Nothing at 5.0				
9	*N*-Dichloroacetyl-β-hy-droxyphenylalanine	Nothing at 3.0				
10	1-Phenyl-2-dichloroacet-amido-1,3-propanediol	0.033		0.039	0.039	Non-competitive and very slight
11	*N*-Dichloroacetyl-β-hy-droxy-*p*-nitrophenyl-alanine	2.0			3.0	Non-competitive and slight
12	1-(*p*-Nitrophenyl)-2-amino-1,3-propanediol	0.30		0.35	0.33	Practically non-existent

* Expressed as the amount (in mg. per cc. of culture) required to cause half
maximal inhibition of growth in the presence of graded concentrations of phenyl-
alanine.

cluded that if Chloromycetin was an antimetabolite of any common amino
acid phenylalanine was the most probable choice.

*Toxicity for Phenylalanine-Requiring Mutant of E. coli of Analogues of
Phenylalanine, and Its Antagonism with That Amino Acid*—The toxicity
of each substance was determined by addition of graded amounts of a
neutral aqueous solution to a series of tubes containing 10 cc. of the glu-

cose-salts medium (9). After sterilization at 120°, inoculation with the mutant strain of *E. coli* was carried out in a manner just described for the wild strain of this organism. All tubes were incubated at 37° for 18 to 20 hours, and growth in each was then determined quantitatively in the photometer. The amount of each compound needed to reduce growth to half maximum was then estimated from a dose-response curve. These values were determined in the presence of various concentrations of phenylalanine in the basal medium. The results are summarized in Table III. Inspection of these data readily showed whether antagonism with the metabolite existed, and if so, whether it was of the competitive kind.
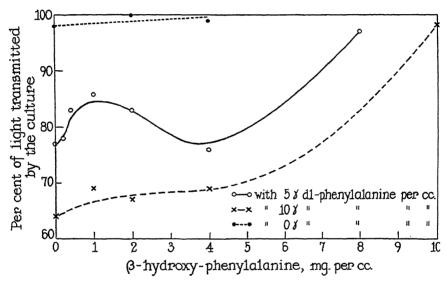
FIG. 2. Responses of the phenylalanine-requiring strain of *E. coli* to β-hydroxy-phenylalanine in the presence of suboptimal and of adequate concentrations of the metabolite.

Toxicity of Analogues for Wild Strain of E. coli—The experiments on toxicity of the compounds were repeated in the way described above, except that the wild strain of *E. coli*, which did not need phenylalanine as a growth factor, was used. The results are summarized in Table IV. In general, the values obtained with the wild strain with no added phenylalanine were comparable to those for the mutant strain in the presence of 20 γ of the metabolite per cc. However, the behavior with β-hydroxy-phenylalanine was qualitatively different.

Anomalous Behavior of β-Hydroxyphenylalanine with Mutant Strain—When β-hydroxyphenylalanine was tested with amounts of phenylalanine

which by themselves were suboptimal for growth, an inhibition of growth was seen, but, as the concentration of the analogue was raised, this gave way to an apparent stimulation. For this reason, no adequate value for the toxicity of this substance in the presence of suboptimal amounts of phenylalanine could be determined. A typical dose-response curve is shown in Fig. 2. This effect was observed only when the concentration of the metabolite was below that needed for optimal growth. Furthermore, it was never found with the wild strain of *E. coli*, or with other wild bacteria which showed a nutritional need for phenylalanine (*e.g. L. casei*).

DISCUSSION

The results of this study indicate that Chloromycetin is a naturally occurring analogue of phenylalanine, and may owe its antibacterial properties to interference with the action of that metabolite. The structural resemblance is unquestionable, but the uncertainty arises from the fact that the antagonism which can be demonstrated is non-competitive. A further uncertainty appears with *E. coli* in that not only phenylalanine, but also tyrosine or tryptophan, exhibits such structural resemblance and biological interaction with the drug. However, this latter effect may be ascribed to the participation of these amino acids in phenylalanine metabolism, because in organisms such as *L. casei*, which are known to lack the ability to produce tyrosine and tryptophan from phenylalanine, only this last substance showed the interference with the action of Chloromycetin.

The major uncertainty in the interpretation then resides in the lack of *competitive* antagonism, and for this reason the examination of analogues intermediate between Chloromycetin and phenylalanine is of interest. For this study, the use of phenylalanine-requiring strains was important, because, as is well known (2), many antimetabolites affect only those species which require the metabolite as a growth factor. The results with the mutant of *E. coli* were similar to those with other phenylalanine-dependent organisms' such as *L. casei*. They were in contrast with some of the findings with the wild (phenylalanine-independent) strain of *E. coli*, because with the latter the fact that several of the analogues were actually competitive antagonists of phenylalanine did not appear. (Compare Compounds 10, 11, and 12 in Tables III and IV.)

With these phenylalanine-dependent organisms, one can see the emergence of non-competitive antagonism as certain structural features were introduced into the metabolite molecule. Four analogues are possible in which just one of the structural alterations linking Chloromycetin to phenylalanine has been made (Compounds 1 to 4 in Table III). The introduction of the dichloroacetyl group alone did not give rise to an anti-

metabolite, but, instead, resulted in a substance which still retained metabolite potency. The reduction of the carboxyl group to a primary alcohol, as in Compound 2, destroyed the metabolite potency of phenylalanine, but was not a great enough change to convert it into an antimetabolite. Likewise, the introduction of the nitro group had a similar result. For the wild strain of *E. coli* both of these substances had a weakly toxic action, which was influenced only slightly by additions of phenylalanine. Of the four possible single alterations, only that yielding β-hydroxyphenylalanine gave an antimetabolite which competed with phenylalanine. When two structural changes were made simultaneously, as in Compounds 6 to 9, only harmless or weakly toxic materials resulted. When three alterations were made simultaneously, the products were competitive antagonists at low concentrations, and completely irreversible inhibitors at higher concentrations. Compounds 10, 11, and 12 will illustrate this. These were the most potent substances encountered in the passage from the metabolite towards the antibiotic agent. Finally, four changes made simultaneously, giving rise to Chloromycetin, yielded a substance which was more potent and less readily reversible in its action by phenylalanine than the preceding analogues.

Two features come to notice as the activities of this series of compounds are scrutinized. (1) Non-competitive antagonism seems to arise when nitro or dichloroacetyl groups are introduced into the molecule. Perhaps these allow the substance to be fixed irreversibly to the site of action of phenylalanine. A tendency to non-competitive behavior may be discerned as well with an increase in the number of structural alterations of the metabolite regardless of their kind. (2) Potency seems to increase as irreversible antagonism emerges. This point is not beyond equivocation, since several of the analogues were weakly toxic even though they were non-competitive with phenylalanine. Furthermore, since the activity of some of the analogues was not influenced at all by phenylalanine, it is quite possible that their toxicity was not concerned with this metabolite.

If Chloromycetin is a naturally occurring antimetabolite of phenylalanine, one should not be surprised to find that it is a non-competitive one. In several other instances in which nature has produced an antimetabolite, as in the case of 3,3'-methylenebis-(4-hydroxycoumarin) (13), difficulty in antagonism by the related metabolite is a prominent feature. With antibiotic agents, the advantage to the producing organism of such a property is obvious, for the activity of the compounds would then not be at the mercy of a mere increase in concentration of the related metabolite in victimized species. In fact, in drugs such as Marfanil one sees a tendency on the part of biochemists to strive for irreversibly active agents.

SUMMARY

The antibacterial powers of Chloromycetin were overcome by additions of phenylalanine to a glucose-salts medium in which *Escherichia coli* was grown. The antagonism was non-competitive and demonstrable only in the presence of minimally effective concentrations of the drug. Although most other amino acids did not exert such an effect, tyrosine or tryptophan did so. With organisms such as *Lactobacillus casei*, only phenylalanine was able to influence the toxicity of Chloromycetin.

All of the compounds intermediate in structure between phenylalanine and Chloromycetin were examined for toxicity to bacteria, and the poisonous ones were tested with graded concentrations of phenylalanine to determine the existence and nature of the antagonism. Results which were most understandable were obtained with organisms which showed a nutritional requirement for the amino acid. With progressively greater structural alteration, a non-competitive and frequently completely irreversible type of antagonism emerged. These results were viewed in relation to the nature of non-competitive antagonism among structural analogues.

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ANTIFATTY LIVER ACTIVITY OF CRYSTALLINE TRYPSIN IN INSULIN-TREATED DEPANCREATIZED DOGS*

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The fatty liver that develops in insulin-treated, depancreatized dogs and in dogs subjected to ligation of their pancreatic ducts has been the subject of considerable study (1). Soon after its discovery it was shown to be prevented by the feeding of raw pancreas (2), while later findings revealed that it could also be prevented by the feeding of pancreatic juice and certain fractions derived from raw pancreas (1).

In understanding this type of fatty liver, it is important to recognize that the condition develops in completely depancreatized dogs fed abundant amounts of choline and methionine in the form of lean meat (1, 3, 4). But the feeding of extra choline or extra methionine, in their free forms, did prevent the development of a fatty liver (4). This difference in the action of free and bound methionine suggested that, in dogs deprived of the external secretion of their pancreases, there is some disturbance in the gastrointestinal tract whereby the methionine of ingested protein is not made available for lipotropic purposes. The subsequent observation that hydrolyzed casein prevented the development of fatty livers, whereas unhydrolyzed casein had no such effect, lent further support to such a view (5).

On the basis of the above findings it was proposed as a working hypothesis that the proteolytic enzymes contained in pancreas accounted for its antifatty liver activity. Evidence for this hypothesis is presented here. It is shown that the feeding of crystalline trypsin effectively prevents the development of fatty livers in completely depancreatized dogs maintained with insulin.

EXPERIMENTAL

For several weeks before pancreatectomy, the dogs were fed a diet high in lean meat and adequate in calories, vitamins, and salts. Only those that showed a vigorous appetite for meat were selected for pancreatec-

tomy. After pancreatectomy, each dog was fed twice daily a mixture containing 250 gm. of lean meat, 50 gm. of sucrose, 5 gm. of bone ash, and 1.5 gm. of salt mixture.[1] Once daily, vitamin supplements[2] were fed along with the morning meal. 8 units of insulin were injected at each time of feeding. For the first 3 weeks after pancreatectomy, 25 gm. of raw pancreas were added to each dietary mixture. This preliminary feeding of pancreas was adopted to insure that at the time the feeding of

TABLE I

Effects of Trypsin Feeding on Fatty Acid Content of Liver of Insulin-Treated Depancreatized Dogs

Dog No.	Weight		Trypsin fed		Liver	
	Preopera-tive	Final	Per meal*	Assay period	Weight	Total fatty acids
	kg.	*kg.*	*mg.*	*wks.*	*gm.*	*per cent wet weight*
D646	9.5	7.2	None	20	670	21.5
D662	6.0	5.5	"	24	580	13.3
D751	7.9	6.5	"	19	310	37.5
D757	9.2	8.2	"	12	720	17.2
D621	12.2	8.0	10†	20	470	2.6
D624	12.0	8.3	10	20	483	3.1
D753	8.3	8.5	10	18	402	2.2
D754	10.8	9.2	10	18	488	2.2
D755	11.5	11.5	10	18	430	2.9
D758	8.0	6.3	10	18	210	2.4
D748	10.5	12.0	5	21	403	2.1
D750	7.0	6.0	5	21	402	1.9

* Each animal received two meals per day.

† The values shown in this column are the mg. of crystalline trypsin present in the amounts of the $MgSO_4$ mixture fed.

trypsin was begun the animal's liver was free of abnormal amounts of fat (6).

The assay period (Table I) began with the removal of pancreas from

[1] This amount of salt mixture contained 600 mg. of NaCl, 450 mg. of Mg citrate, 200 mg. of KH_2PO_4, 130 mg. of $CaHPO_4 \cdot 2H_2O$, 30 mg. of ferric citrate, 0.1 mg. of KI, and 115 mg. of KCl.

[2] 10 cc. of Galen "B" and 3 cc. of a fish oil were used. Each cc. of the former contained 0.15 mg. of thiamine, 0.15 mg. of riboflavin, 2.0 mg. of niacin and niacinamide, 0.15 mg. of pyridoxine, 0.4 mg. of pantothenic acid, 7.2 mg. of inositol, and 0.001 mg. of biotin. Each cc. of the fish oil contained 400 A. O. A. C. chick units of vitamin D and 1000 U. S. P. units of vitamin A.

the diet, and lasted for 18 to 20 weeks. The trypsin used was Armour's crystalline preparation containing 50 per cent $MgSO_4$. The dry mixture was added to the diet just before feeding. Six dogs received daily 10 mg. of crystalline trypsin; *i.e.*, 20 mg. of the mixture with each meal. Two other dogs received 10 mg. of the trypsin mixture with each meal.

Four dogs served as controls; the dietary treatment of these four dogs was identical with that of the other eight, except that they received no trypsin.

At the end of the assay period, the dogs were anesthetized with nembutal and their livers were excised. Each whole liver was thoroughly ground and mixed, and a sample of the mixture was taken for determination of total fatty acids (7).

Results

In order to assess the antifatty liver activity of trypsin, trypsin was fed twice daily for 18 to 20 weeks. The reasons for extending the assay period to this length of time, as well as the validity of the assay procedure used here, have been discussed elsewhere (6).

The results shown in Table I clearly demonstrate the effectiveness of trypsin, added to the diet, in preventing the accumulation of fat in the liver of the insulin-treated, depancreatized dog. The fatty acid contents of the livers of the eight dogs that received 5 or 10 mg. of trypsin per meal did not exceed 3.1 per cent. The livers of the four control dogs were uniformly fatty, and contained from 13 to 37 per cent fatty acids.

The possibility that trypsin might have an effect on lipide metabolism was also recognized recently by Canepa *et al.* (8). These investigators fed this enzyme to insulin-treated, depancreatized dogs for 1 week and found no response in the blood lipides. This extremely short feeding period precluded, of course, a precise determination of the antifatty liver activity of this substance.

DISCUSSION

Lean meat or casein, even when fed in very large amounts, fails to prevent the development of fatty livers in insulin-treated, depancreatized dogs. Although the usual daily ration contained 500 gm. of lean meat, fatty livers have also been observed in dogs that were fed as much as 1120 gm. of lean meat per day (3). The most striking example of the failure of protein to exert its normal lipotropic effect was observed in a study in which depancreatized dogs were fed, daily, 500 gm. of lean meat plus 80 gm. of casein (5); the livers of three dogs fed this diet contained 17 to 22 per cent fatty acids.

But when admixed with trypsin, lean meat does exert its normal lipo-

tropic effect in the insulin-treated, depancreatized dog. It was reported earlier that the feeding of 5 cc. of pancreatic juice along with the lean meat diet also prevented fatty livers in these dogs and it would appear that the tryptic activity of this juice may account for its antifatty liver property. These observations are, of course, consistent with the view that ascribes the development of fatty livers in the insulin-treated, depancreatized dog to a loss of the external secretion of its pancreas.

The results of the present investigation illustrate an important aspect of the lipotropic action of proteins; namely, its dependence upon the digestive capacity within the small intestine. In this sense, trypsin may be regarded as an intrinsic antifatty liver factor.

It should not be inferred, from the results of this study, that trypsin is the only intestinal enzyme concerned with antifatty liver activity. We have recently observed that the feeding of crude papain also prevents fatty livers in completely depancreatized dogs injected with insulin. Since chymotrypsin and carboxypeptidase may be concerned in the liberation of methionine from proteins, they also should be considered as possible antifatty liver agents. These possibilities are being explored in this laboratory.

SUMMARY

1. Trypsin has been identified as a possible intrinsic antifatty liver factor in the dog. The addition of as little as 5 mg. of trypsin to each lean meat meal fed insulin-treated, depancreatized dogs completely prevented the development of fatty livers.

2. The dependence of lipotropic action of proteins upon the digestive capacity of the intestine is discussed.

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CHEMICAL ANALYSIS OF THE T₇ BACTERIOPHAGE OF ESCHERICHIA COLI*

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Chemical analyses have been made of several bacteriophages (1–9) in more or less purified preparations. However, only a few, the T₂ (6–8) and, more recently, the T₆ (9) bacteriophages of *Escherichia coli*, have been studied in sufficient detail for comparison with analogous studies on plant and animal viruses. The results obtained on the bacteriophages thus far examined have shown that, while these agents are somewhat similar to the animal viruses in the constitution of their major components, the bacterial viruses differ, with few exceptions, from other viruses in the quantitative distribution of components, the difference being manifested principally in an exceedingly high content of nucleic acid in the bacterial viruses. Judging from phosphorus content and direct estimation, the content of nucleic acid has been uniformly high in the various bacteriophages (1–9), and it would appear that this property and certain others may be characteristic of these agents. Recently, studies (10) were undertaken on another bacteriophage, T₇ of *E. coli*, which was chosen because of its apparently great difference from the T₂ agent in size, morphology, and immunological properties (11). The T₇ virus is spherical, has about 12 per cent of the mass of the T₂ (and T₆) phage, has no tail, and is immunologically unrelated to either the T₂ or T₆ agents. The purification and some of the properties of the T₇ bacteriophage were reported in a recent publication (10). The results of chemical analyses of the agent and a comparison with analogous findings with T₂, T₆, and the host organism, *E. coli* (strain B), are described in the present paper.

Materials and Methods

Purification—The T₇ bacteriophage and the host organism *E. coli* (strain B) were obtained from Dr. M. Delbrück. Cultivation of the agent for purification was carried out by the procedures already described (10). Most of the analyses were made on two different batches, A and B, prepared in the present work. Some estimations were made with the phage prepared in the previous experiments.

Batch A was obtained in the period from May 30 to July 14, 1949, from five separate lots of lysates with a total volume of 155 liters. Broth cultures of the bacterium were inoculated with the phage, and the mixture was kept in the incubator at 37.5° until lysis was complete (60 to 75 minutes). The crude lysate was transferred to a cold room at 4° for 10 to 16 days, after which it was passed through 8 pound Mandler filters and concentrated in the Sharples centrifuge in the usual way. The Sharples concentrate was spun in the angle centrifuge at 700 $\times$ *g* and passed through a 14 pound Mandler candle. Further concentration was effected by centrifugation at 26,000 $\times$ *g* in the air-driven centrifuge. The resulting pellets were taken up with vigorous pipetting in Ringer's solution of pH 7.4.

After the ultracentrifuge concentrates from the various lots were pooled, it was found that the preparation was still grossly contaminated with bacteria. The concentrate was then spun in the angle centrifuge for 10 minutes at 900 $\times$ *g*, 10 minutes at 5000 $\times$ *g*, and twice in the horizontal centrifuge, in pointed glass tubes, once for 20 minutes at 1200 $\times$ *g* and once for 30 minutes at 1200 $\times$ *g*. This treatment failed to remove the bacteria, and the concentrate was again spun in the horizontal instrument for 1 hour at 1200 $\times$ *g*. At this point, the bacterial count was $10^{5.11}$ organisms per ml., an amount considered negligible as a factor in analyses on the phage in the preparation containing 0.149 mg. of nitrogen per ml. The infectious unit of phage was $10^{-16.3}$ gm. of N. The concentrate containing 56.2 mg. of phage was dialyzed against distilled water for 60 hours and lyophilized.

Batch B was prepared during the period from July 5 to October 8, 1949, from three separate lots of crude lysate of 146 liters total volume. The procedures were the same as those above, except for a few modifications. The crude lysate was left in the cold for 70 to 120 days before purification was begun. The number of bacteria in the Sharples concentrate was negligible; therefore further purification consisted of spinning the Sharples concentrate once in the angle centrifuge for 10 minutes at 700 $\times$ *g*, filtration of the concentrates through a 14 pound Mandler candle, and concentration of the phage by three cycles of alternate high speed spinning in the ultracentrifuge at 26,000 $\times$ *g* for 1 hour and low speed centrifugation in the horizontal instrument for 10 minutes at 1575 $\times$ *g*. In all cases with this batch, the pellets were taken up with gentle pipetting after standing for a time in the solvent. The bacterial count on the pooled concentrates was less than 10^5 organisms per ml., and the infectious unit of phage was $10^{-16.04}$ gm. of N. The yield was 60.2 mg. of phage after dialysis for 24 hours, and the infectious unit was the same as before. The preparation was lyophilized as usual.

For analysis, the preparations were dried to constant weight over P_2O_5 at 34°.

Analytical Procedures—Nitrogen was estimated by a micro-Kjeldahl method developed in this laboratory,[1] and phosphorus was determined according to King with slight modifications (12). The cysteine-sulfuric acid method of Stumpf (13) was used for the estimation of desoxyribonucleic acid (DNA), and the phloroglucinol-HCl method of Hahn and von Euler (14) was employed for ribonucleic acid (RNA). Commercial thymus nucleic acid (The British Drug Houses, Ltd., London) was used as a standard for DNA and purified yeast nucleic acid for the RNA. In each of the standards, the amount of nucleic acid was calculated on the basis of phosphorus (DNA = phosphorus × 10.1; RNA = phosphorus × 10.6).

Carbohydrate was identified by the partition chromatographic technique described by Partridge (15) and by the absorption spectrum of the pigment resulting from the action of phloroglucinol-HCl. For chromatography, the spots were placed on sheets of Whatman No. 1 filter paper. A butanol-acetic acid mixture or *s*-collidine was used as solvent, and the chromatograms were developed with either silver nitrate-ammonia mixture or phthalic acid-aniline (16). Quantitative determinations of carbohydrate were made by the copper reduction method of Somogyi (17) and by the orcinol method of Sørensen and Haugaard (18).

The Evelyn photoelectric colorimeter was used in all colorimetric procedures, and the Beckman photometer was employed for determining light absorption of the pigment in the phloroglucinol reaction.

Analyses and Results

Analysis of Whole Complex—The general properties of the T_7 phage and the results of qualitative tests on the whole complex were reported in the previous paper (10). Preliminary quantitative analyses showed the presence of N 12.3 and P 3.7 per cent; carbohydrate values, in terms of glucose, were 16.5 and 16.8 per cent by the copper reduction method of Somogyi and the orcinol reaction of Sørensen and Haugaard. Evidence of the presence of glucose and ribose in the complex was obtained by chromatography.

In the present work, the whole complex was analyzed for nitrogen, carbohydrate, and DNA content. For N and DNA determinations, samples of 2 to 4 mg. of the dry phage were kept overnight at 37° in 2 ml. of 5 per cent KOH. The resulting, slightly turbid solutions were made up to 10 ml. with distilled water. Estimations were made on aliquot samples of these solutions. The results are given in Table I, together with Taylor's

[1] Lanni, F., Dillon, M. L., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, in press.

values (6) on T_2 bacteriophage and the results of Kozloff and Putnam (9) with the T_6 bacteriophage. The composition of the host, *E. coli*, is shown also for comparison.

Carbohydrate was determined on 2 to 4 mg. samples of the complex. The samples were placed in Pyrex tubes, 2 ml. of N HCl were added, and the tubes were sealed. After the tubes had been immersed for 3 hours in a boiling water bath, the hydrolysate was neutralized with 4 N NaOH and made up to 25 ml. Samples of 2 ml. were employed for the estimation of reducing capacity and for the orcinol determination. The carbohydrate values of Table I, expressed as glucose, are the means of the results with the two methods.

TABLE I

Chemical Composition of T_7 Bacteriophage Compared with Composition of T_2 and T_6 Bacteriophages and of E. coli Cultured in Broth*

	T₇		T₂†	T₆‡	E. coli†
	Batch A	Batch B			
Nitrogen	14.73	11.50	13.5	13.3	13.2
Protein	48.65§	36.70§	50.6		67.9
Phosphorus	4.18	3.46	4.84	3.95‖	2.72
Carbohydrate	16.60	31.1	13.6		12.5
Desoxyribonucleic acid	41.10	33.3	40.3	42.0	5.2

* The values represent percentage of dry weight of the whole complex.
† Data from Taylor (6).
‡ Data from Kozloff and Putnam (9).
§ Protein = (total nitrogen) − (nucleic acid nitrogen) × 6.25. See foot-note 2.
‖ Personal communication of Dr. L. Kozloff.

The total phosphorus of the whole complex, given in Table I, was calculated from the results of analyses on the fractions described below and shown in Table II. Protein (Table I) was calculated[2] as the product of the factor 6.25 × (total *N*-nucleic acid N).

Identification of Carbohydrates—Chromatographic analysis was made on samples of 1 to 2 mg. of dry phage hydrolyzed with 2 ml. of N HCl or 0.5 N H_2SO_4 in sealed tubes immersed in a boiling water bath for 3 to 6 hours. The hydrolysates were chilled, those obtained with H_2SO_4 were neutralized with $Ba(OH)_2$, and all were concentrated *in vacuo* and taken up in distilled water. The water was driven off, and the residue, taken up in a

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[2] The factor 6.25 is probably too large in view of the high nitrogen content of the protein usually combined with nucleic acid. In the absence of direct studies on the character of the protein of the bacteriophages, the conventional factor was employed to obtain an approximation of protein content.

very small amount of water, was put on filter paper. Fig. 1 shows the tracings of some typical chromatograms. Glucose was detected in all of the hydrolysates. Desoxyribose was never found, possibly because it was destroyed during the hydrolysis. In a single case, a second weak reducing spot was seen, the position of which corresponded to ribose.

The phloroglucinol-HCl test for RNA was carried out with Batches A and B, and like tests were made also with pure ribose and glucose and with

TABLE II

Distribution of Nitrogen (N), Phosphorus (P), Desoxyribonucleic Acid (DNA), and Carbohydrate (CH) in Fractions of T_7, T_2, and T_6 Bacteriophages and of E. coli*

| | | T_7 | | T_2† | T_6‡ | *E. coli*‡ |
		Batch A	Batch B			
Fraction I	N	2.7	2.5			
	P	0.7	6.6		3.2	20.7
	DNA	0.0	1.0			
	CH	22.0	20.1			
Fraction II	N	1.3	1.0			
	P	1.0	2.4	0.0	1.5	11.1
Fraction III	N	48.9	48.5			
	P	94.5	87.9	92.7	90.5	61.6
	DNA	97.8	99.0			
	CH	56.3	76.4			
Fraction IV	N	47.1	48.0			
	P	3.8	3.1	5.3§	4.8	5.2
	DNA	2.2	0.0			
	CH	21.7	3.5			
Petroleum ether-soluble	Total	1.0		2.6		7.6†
	N	0.0	0.0	0.0		0.2†
	P	0.0	0.0	0.0		0.3†

* The values represent percentage distribution of the total phage constituents.
† Data from Taylor (6).
‡ Data from Kozloff and Putnam (9).
§ Inorganic P included.

mixtures of glucose and ribose. The resulting absorption spectra of the colored products are given in Figs. 2 and 3.

In Fig. 2 there are shown the absorption curves of (1) glucose, (2) a mixture containing 90 per cent glucose and 10 per cent ribose, and (3) a mixture containing 50 per cent glucose and 50 per cent ribose. There are shown, for comparison, the absorption curves of the pigment resulting from the action of the phloroglucinol reagent on Batches A and B of the T_7 bacteriophage. In Fig. 3 there is given the comparison of the curves ob-

tained with (1) a mixture of 10 per cent glucose and 90 per cent ribose and (2) ribose together with the curve (C) obtained with *E. coli*.

It is seen in Fig. 2 that the curves obtained with the virus fall in the region intermediate between those observed with mixtures of glucose and ribose. In a like manner, the absorption spectrum with *E. coli* was similar to that of a mixture of glucose and ribose containing principally the latter sugar. Calculations were made to estimate the distributions of glucose
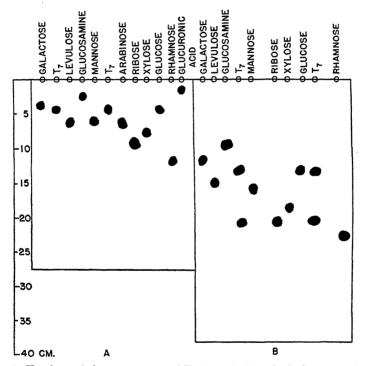
FIG. 1. Tracings of chromatograms of T₇ bacteriophage hydrolysates and of pure sugars. The chromatograms of *A* were obtained with butanol-acetic acid solvent and those of *B* with *s*-collidine. The respective base-lines of *A* and *B* represent the solvent fronts.

and ribose in T₇ bacteriophage and in *E. coli* by taking the values read at 460 mμ and 630 mμ, respectively, the molecular extinction of both glucose and ribose at the same wave-lengths being known. The values are probably only approximations, since there are differences between the shapes of the curves obtained with the pure carbohydrates and those representative of the T₇ bacteriophage and the host bacterium. The values were as follows: T₇, Batch A, 77 per cent glucose and 23 per cent ribose, corre-

sponding to 3.8 gm. of ribose per 100 gm. of virus.   T$_7$, Batch B, 88 per cent glucose and 12 per cent ribose, corresponding to 2.7 gm. of ribose per 100 gm. of virus; *E. coli*, 15 per cent glucose and 85 per cent ribose.

Fractionation—Fractionation of the bacteriophage was effected by the trichloroacetic acid method of Schneider (19).   Quantities of 10 to 20 mg. of the dry virus were stirred with 2.5 ml. of cold, 10 per cent trichloroacetic
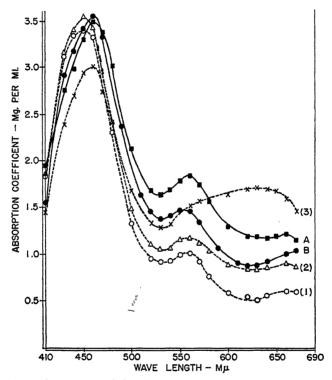
Fig. 2. Absorption spectra of the pigment resulting from the action of phloroglucinol reagent on the two batches of T$_7$ bacteriophage, Curves A and B; on glucose, Curve 1; on a mixture of 90 per cent glucose and 10 per cent ribose, Curve 2; and on 50 per cent glucose and 50 per cent ribose, Curve 3.

acid.   The pellet obtained by centrifugation was extracted in the same way with a second 2.5 ml. volume of 10 per cent trichloroacetic acid.   The combined extracts were made up to 5 ml. (Fraction I).

The insoluble residue was extracted with a mixture of 1 ml. of water and 4 ml. of 95 per cent ethyl alcohol, followed by three further extractions with 5 ml. each of cold alcohol-ether (3:1).   The combined extracts were made up with alcohol-ether to 25 ml. (Fraction II).

The remaining residue was taken up in 1 ml. of water; 1.5 ml. of 10 per cent trichloroacetic acid was added, and the preparation was centrifuged. The insoluble material was stirred up in 5 ml. of 5 per cent trichloroacetic acid, heated for 15 minutes in a water bath at 90°, and centrifuged. The pellet was taken up again in 5 ml. of 5 per cent trichloroacetic acid, kept overnight in an incubator at 37° and then in a water bath at 90° for 15 minutes, and centrifuged. Finally, the residue was washed with 2.5 ml. of cold 5 per cent trichloroacetic acid (Fraction III). The residue was dissolved, in a boiling water bath, in 5 ml. of 2 per cent NaOH (Fraction IV).
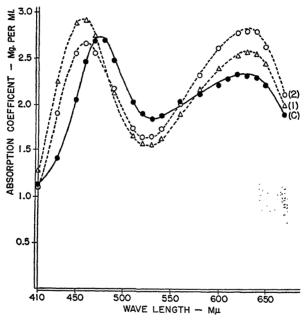
FIG. 3. Absorption spectra of the pigment resulting from the action of phloroglucinol reagent on a hydrolysate of *E. coli*, Curve C; on 10 per cent glucose and 90 per cent ribose, Curve 1; and on ribose, Curve 2.

The alcohol-ether fraction (Fraction II) was further treated as follows: About 20 ml. of the solution were evaporated in an oven at 57°, and the residue was extracted with petroleum ether which had been previously purified by washing with concentrated sulfuric acid and redistillation. The petroleum ether solution was filtered and made up to 10 ml.

The trichloroacetic acid and petroleum ether fractions were analyzed for nitrogen, phosphorus, DNA, and carbohydrate. The values given in Table II represent the percentage of the total amount of the constituents of the whole complex present in each of the fractions.

DISCUSSION

The results given in Tables I and II show the remarkable similarity of T_7 bacteriophage to the other two $E.$ $coli$ phages already analyzed, namely, the T_2 and T_6 phages (6–9). All three of these agents seem to contain nucleic acid in large amounts (about 40 per cent) besides protein, carbohydrate, and lipide. Little was done with the lipide of the T_7 agent since the petroleum ether-soluble material was small in amount, less than 1 per cent of the total dry material. Because of its small size, the phage could be obtained only in small quantities and then only with considerable effort. It seems, however, that about 1 to 2 per cent of the total phosphorus and about 1 per cent of the total nitrogen of T_7 bacteriophage are alcohol-ether-soluble. Taylor (6) did not detect phosphorus in the alcohol-ether fraction, while Kozloff and Putnam (9) found about 1.5 per cent of the total phosphorus in the alcohol-ether fraction of the T_6 bacteriophage.

There was a notable difference between the chemical compositions of the two batches, A and B, analyzed in the course of this work, the sugar content of the latter being about twice as great as that of the former. The findings with Batch A were identical with those of the material analyzed in the previous work (10). The phloroglucinol estimation made here with each trichloroacetic acid fraction showed that more than 50 per cent of the carbohydrate passes into the hot trichloroacetic acid fraction (Fraction III) but that about 20 per cent can be extracted by treatment with cold trichloroacetic acid (Fraction I). It is possible that a part of the carbohydrate of Batch B bacteriophage originated from the host organism. When a suspension of $E.$ $coli$ is inoculated with T_7 bacteriophage, lysis occurs at 37° within 60 to 75 minutes, but the crude lysate is hard to filter because of a mucin-like material originating most probably from the bacteria. On keeping the lysate in the cold for several weeks, the mucoid material gradually disappears. It is possible that the bacteriophage particles absorbed some polysaccharide-like breakdown product of the mucoid material of the $E.$ $coli$. This hypothesis might explain the larger amount of carbohydrate in Batch B, since this preparation was kept for a longer time in the refrigerator. Furthermore, in the process of purification, resuspension of the pellets obtained by ultracentrifugation was more gentle. It is probable that the lower sugar contents found in the previous work (10) and in Batch A represent the properties of T_7 which are more comparable to the findings with T_2 with respect to the conditions of preparation of the respective agents.

As in the instances of the T_2 and T_6 bacteriophages, the question arises of the presence or absence of RNA in the T_7 bacteriophage. Taylor (6) reported that about 10 per cent of the nucleic acid in the T_2 bacteriophage was RNA. Cohen (7, 8) was unable to find any evidence for the presence of RNA in the T_2 bacteriophage. Kozloff and Putnam (9) found that

about 7 per cent of the nucleic acid phosphorus of the T_6 phage was present as RNA. The phloroglucinol test of Hahn and von Euler used for estimation of RNA gives a green color with ribose as well as with other pentoses. The absorption curves with pentoses (Fig. 3) show a strong absorption in the blue region at 460 mμ and a second strong absorption in the red region at 630 mμ. The hexoses also give a color with the phloroglucinol reagent, but the pigment shows very strong absorption in the upper spectral range, as illustrated by glucose in Fig. 2, and the color is brown. If ribose is mixed with glucose, the resulting color depends on the ratio of glucose to ribose, but even 10 per cent ribose in the mixture changes the shape of the absorption curve, giving higher absorption in the red range. The higher the percentage of ribose, the higher is the absorption maximum at 630 mμ. The T_7 bacteriophage gives a brown color with the phloroglucinol reagent, but the spectrographic analysis shows that the curve corresponds rather to a mixture of glucose and ribose than to that of glucose alone.

The paper chromatographic analysis gave another evidence for the presence of ribose in the T_7 bacteriophage. The pentose could not be detected in all of the hydrolysates, a result which could be due to the relatively small amounts of ribose in the bacteriophage and to the difficulties of splitting off all of the ribose bound to pyrimidine bases. No systematic study has been made in this respect, but in the single case in which ribose was revealed, hydrolysis had been effected by weak sulfuric acid.

It would appear that ribose is certainly present in the T_7 bacteriophage. The experiments, however, did not answer the question of whether the ribose is present in the form of ribonucleic acid. The phosphorus values correspond fairly well to the amount of DNA alone, assuming that only DNA is present in the virus. One has to take into consideration, however, that the estimation of the DNA involves errors since, usually, thymus nucleic acid is used as the control, and its composition may differ from that of the nucleic acids in the T_7 bacteriophage. Final judgment regarding the presence or absence of RNA in these bacteriophages must await a more thorough study with adequate amounts of demonstrably homogenous material.

Despite the apparently wide differences between the T_7 phage and the T_2 and T_6 viruses, as seen in their physical, morphological, and immunological properties, no fundamental differences were seen in their chemical constitutions. Consequently, the experiments with the T_7 phage add one more agent to the group of bacterial viruses having properties in common with respect to chemical composition (within the scope of examinations made). In the high content of nucleic acid, the phages differ from their bacterial host organisms, from the animal viruses studied, and from the

plant viruses, with the exception of the tobacco ringspot agent. The large amount of nucleic acid, however, is comparable with that of such cellular elements as sperm and thymus gland cells (20, 21).

SUMMARY

Chemical analyses have been made of the purified T_7 bacteriophage of *E. coli*. The virus consists of nucleic acid about 40 per cent, protein about 50 per cent, and lipide about 1 per cent. The carbohydrate content of two batches of the virus, one analyzed in a previous study, was about 16 per cent. Another sample of the agent contained carbohydrate to the extent of about 30 per cent. The presence of glucose and ribose was demonstrated in hydrolysates of the T_7 phage by means of chromatography and the color reaction with phloroglucinol-HCl. The constitution of the T_7 phage was similar to that of the T_2 and T_6 bacteriophages of *E. coli*.

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THE INACTIVATION OF INVERTASE BY TYROSINASE

I. THE INFLUENCE OF CERTAIN PHENOLIC COMPOUNDS ON THE INACTIVATION*

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The oxidation of tyrosyl groups in certain proteins by tyrosinase has been previously reported (Sizer (1, 2)), as well as the partial inactivation of yeast invertase (3), and indirect evidence suggested that loss of activity was associated with oxidation of tyrosyl groups in the invertase molecule. Results on invertase were puzzling, however, since it was reported that crude tyrosinase, although high in activity, was inert toward invertase, while one tyrosinase of high purity (catecholase) was effective in the inactivation of invertase, and another equally pure (cresolase) was inert toward invertase. The failure of the crude preparation to inactivate invertase might find an explanation in the presence in the impure tyrosinase preparation of tyrosinase substrates which are known to be present and account for a gradual darkening of the tyrosinase preparation (4), and which might competitively prevent action of the tyrosinase on the invertase. The failure of certain purified preparations such as cresolase to show an effect cannot be explained on the basis of any fundamental difference between catecholase and cresolase, since there appears to be none (Mallette and Dawson (5)). Possibly some mediator or activator necessary for the union of tyrosinase with invertase is removed during certain kinds of purification procedures.

The present investigation was undertaken with the purpose of resolving the problems raised in the preliminary report on the inactivation of invertase by tyrosinase (3) and of obtaining additional information on the general problem of the inactivation of proteins by tyrosinase.

Methods

Tyrosinase Preparations—Tyrosinase was prepared from mushrooms by the method of Dalton and Nelson (6), according to Jensen and Tenenbaum (7), by the recent method of Mallette *et al.* (8), and from potatoes according to Kubowitz (9). We have also used highly purified tyrosinase[1]

and numerous commercial preparations (Treemond, Worthington, and Synzyme). Although the activity varied considerably from one tyrosinase preparation to the next, a typical one contained about 1500 Miller-Dawson units (4) and 1 mg. of dry weight per ml.

Invertase Preparations—It seems to make no difference what invertase is used; both Wallerstein and Difco preparations are satisfactory. Most of the work was done, however, with a very highly purified yeast invertase[2] (10).

Standard Technique—In a typical experiment, 0.5 ml. of tyrosinase is incubated with 0.1 ml. of diluted invertase, 0.5 ml. of 0.05 M phosphate buffer, pH 6.0, and 0.5 ml. of water plus 0.1 ml. of toluene for 18 hours at 37°. A control experiment, run simultaneously, is identical except for the fact that the tyrosinase has been inactivated by boiling.

In many experiments an additional anaerobic control was run which is identical with the experimental except that nitrogen is bubbled through the solution in a Thunberg tube and the gas is then removed with a high vacuum pump. The anaerobic control usually checks very well with the control containing boiled tyrosinase, except under exceptional conditions not related to the present problem.

Results

Typical results encountered in several hundred different experiments are illustrated in Fig. 1. The tyrosinase solutions were catecholase and cresolase preparations of high purity and activity from Dr. C. R. Dawson. This particular catecholase preparation yielded consistently positive results for the inactivation of invertase. In the experiment of Fig. 1, middle curves, the invertase oxidized by tyrosinase had only 63 per cent of the activity of the control invertase. On the other hand, cresolase consistently failed to inactivate the invertase (upper curve). Rather surprisingly, however, if this active catecholase preparation was dialyzed before use, or if it was diluted to one-fifth of its original strength, the tyrosinase became inactive toward invertase (see lower curve) but not inactive toward typical substrates such as catechol or tyrosine.

In general, crude tyrosinase was inert toward invertase, as were certain highly purified preparations. In the purification of certain catecholase fractions, as they became more active toward catechol they lost their activity toward invertase. Commercial samples varied but preparations received in 1947 (of somewhat lower purity) were consistently more active

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[2] The author is most grateful to Dr. Hector Dieu, University of Liège, for a generous sample of high purity invertase. The invertase solution had a time value of 0.30 minute and contained 0.20 mg. of nitrogen and 0.40 mg. of carbohydrate per ml. It was diluted 250 times before use.

toward invertase than those received in 1949. The variability in effectiveness of the different tyrosinase preparations, and the loss in activity on dilution or dialysis and frequently by elaborate purification techniques, all pointed to the likelihood that there was some factor in the mushroom extracts which was necessary for, or at least greatly facilitated, the action of tyrosinase on invertase.
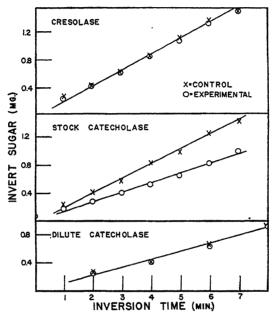
FIG. 1. The hydrolysis of sucrose by invertase is plotted as a function of digestion time. Upper curve, invertase had been treated for 18 hours with active cresolase (experimental) or with boiled cresolase (control); middle curves, same but catecholase was used; lower curve, same as middle curves, except that only 0.2 as much catecholase was used.

It has been known for some time that the oxidation of certain refractory substrates by tyrosinase is tremendously accelerated by the addition of very small amounts of phenolic derivatives. For example, the oxidation of monophenols by tyrosinase from mushrooms is greatly increased by minute amounts of catechol (4), and the oxidation of tyrosine by tyrosinase from mouse melanoma is very responsive to the presence of a small amount of dopa (11). The possibility that some such substrate as catechol or dopa might play a rôle as an activator or intermediate (mediator) in the tyrosinase-invertase system did not seem unlikely, since crude tyrosinase is

known to contain one or more substrates which are gradually oxidized and cause the enzyme solution to darken (4).

In order to test the action of phenolic derivatives on the tyrosinase-invertase system, the reaction was run in the usual way with a sample of Treemond tyrosinase previously found to be inert toward invertase. 1.0 to 0.001 mg. of phenolic compound was added to both the experimental and control digests (final volume, 1.6 ml.) and the system was incubated for 18 hours at 37°. With certain phenols (see Fig. 2) the effect was a marked
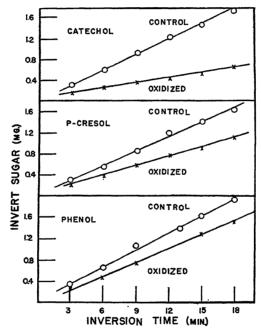
Fig. 2. Invertase activity after previous oxidation of the invertase by tyrosinase plus a phenolic activator. Boiled tyrosinase plus phenolic activator was used in the control.

one, resulting in a very appreciable inactivation of the invertase by the tyrosinase. The inactivation was more extensive and occurred at a much more rapid rate than ever occurs without the addition of a phenolic activator. In a typical experiment (Fig. 3) with 0.1 mg. of catechol in the digest, the inactivation follows the course of a first order reaction for a considerable period following a brief lag phase. The final retardation of the reaction (Fig. 3, Curve 1) apparently reflects the exhaustion of the phenolic activator by its oxidation to a pigment, since in a second experiment (Fig. 3, Curve 2), in which the catechol was added in 0.01 mg. quanti-

ties every 5 minutes until 0.1 mg. had been added, the inactivation was much more extensive. The addition of 1 mg. of catechol instead of 0.1 mg. causes the inactivation of invertase by tyrosinase to proceed practically to completion.

It is apparent from Fig. 2 that catechol is not the only activator of the tyrosinase-invertase system; *p*-cresol and phenol are effective as also are tyramine and epinine. Without effect on this system were tyrosine, dopa, adrenalin (which strongly inhibits invertase), thyroxine, insulin, salicylate, casein hydrolysate, and a crude extract of mushrooms.

Three explanations for the above results present themselves. (1) The phenolic activators are oxidized by tyrosinase to melanin-like end-products, and it is these melanin-like end-products which inactivate the invertase.
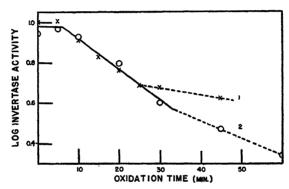
FIG. 3. The inactivation of invertase by tyrosinase plus 0.1 mg. of catechol follows first order kinetics, after a brief lag period, until all the catechol is oxidized. For Curve 1 the catechol was all added at the beginning, but for Curve 2 the catechol was added in ten 0.01 mg. portions at 5 minute intervals.

(2) It is the quinones resulting from the partial oxidation of the phenolic activators which combine with the invertase to form an inert complex. (3) The phenolic activators act as true coenzymes, presumably by their reversible oxidation to quinones or semiquinones which in turn oxidize the invertase, the quinones then being reduced back to phenol or semiquinones.

The first possibility was studied by oxidizing the typical activator with tyrosinase for varying periods of time, inactivating the tyrosinase by heating, and then testing the effect of the oxidation products on the invertase In no experiment did the end-products of catechol oxidation inhibit the invertase.

Since it is well known (4) that phenols in their oxidation by tyrosinase to a melanin-like polymer pass through a quinone intermediate, one method of testing the second hypothesis was to study the possible inhibition of

invertase by quinones. The addition of 0.1 ml. of saturated p-quinone to 0.9 ml. of water, 0.5 ml. of 0.05 M phosphate buffer, pH 6.0, plus 0.1 ml. of invertase (diluted 250 times) resulted in a 26 per cent loss in invertase activity when held at 37° for 18 hours, as compared with the control which contained no p-quinone. A similar experiment in which 1 mg. of o-quinone was used resulted in a 44 per cent inactivation.

While these experiments indicate that the formation of quinones toxic to invertase could explain the partial inactivation of invertase by tyrosinase in the presence of certain phenolic activators, this work does not exclude the possibility that their major action is as coenzymes. If this were the case, the interaction between enzyme, coenzyme, and substrate would be an intimate and direct one, and could not occur if tyrosinase and invertase were separated.

The coenzyme hypothesis is not the correct one, however, for the following experiments demonstrate that when invertase is separated from the system by a cellophane membrane it can still be inactivated by tyrosinase plus certain phenolic activators. To test the coenzyme hypothesis, the experiment (containing active tyrosinase) and the control were set up in the usual way, each solution containing added phenolic activator. Instead of running the two experiments in separate tubes, the control was placed in a small cellophane dialysis sac which was floated in the experimental solution. To facilitate diffusion between the two solutions the tube was constantly shaken during the experiment. A separate control was also run simultaneously in a dialysis sac immersed in a buffer solution containing phenolic activator but no tyrosinase. If the invertase dialyzed against tyrosinase plus phenolic activator showed a lower activity than the invertase dialyzed against the activator alone, it could be concluded that in the former instance the tyrosinase has oxidized the activator to a dialyzable intermediate which diffuses across the membrane and inactivates the invertase.

The results of the dialysis experiments are summarized in Table I. While the toxic, partially oxidized intermediates, produced by tyrosinase action on catechol, p-cresol, and phenol, inactivate invertase by diffusion across a dialysis membrane, the same is not true for epinine or tyramine. Are these compounds true coenzymes or are their toxic oxidation intermediates so unstable as to prevent their access to the invertase molecules some distance away? The latter appears to be the case, since if the life time of the intermediate is increased by lowering the temperature to 0° in the case of tyramine and epinine then the invertase is inactivated. The opposite effect for catechol is obtained by changing the pH from 6.0 to 7.3, thereby rendering the intermediate too unstable to inactivate the invertase some distance away.

All the above data do not find a logical explanation for the inactivation of invertase by tyrosinase in the presence of a phenolic activator in terms of a direct action of the tyrosinase on the invertase, with phenolic activator acting as a coenzyme. Rather, the action seems to be an indirect one; the invertase is inactivated by a quinone resulting from the action of the tyrosinase on the phenolic compound. On this theory one would expect that an inorganic catalyst for the oxidation of phenols would be as effective as tyrosinase in the inactivation of invertase in this system. Studies were therefore made with $FeCl_3$ instead of tyrosinase in the system. The experimental tube contained 0.1 mg. of catechol, 0.5 mg. of $FeCl_3$, 0.5 ml. of acetate buffer, pH 5.0, 0.1 ml. of invertase (diluted 250 times),

TABLE I

Action of Tyrosinase Plus 1 Mg. of Phenolic Activator on Inactivation of Invertase Separated from System by Cellophane Dialysis Sac

Activator	pH	Temperature	Per cent inactivation
		°C.	
Catechol	6.0	37	82
	5.0	37	91
	7.3	37	0
p-Cresol	6.0	37	34
	7.3	37	15
Phenol	6.0	37	99
Tyramine	6.0	37	0
	5.0	37	0
	7.3	37	0
	6.0	0	82
Epinine	6.0	37	0
	6.0	0	13

and 0.8 ml. of water. The control tube was the same except for the omission of catechol. The results are presented in Fig. 4 from which it appears that $FeCl_3$ can satisfactorily replace tyrosinase in this system for the inactivation of invertase by quinones.[3]

While in other studies (1, 2) on the action of tyrosinase on proteins (no phenolic compounds added) only the tyrosyl groups of the proteins were oxidized, it appears from the present investigation that when phenolic activators are present the action is less specific and may involve such groups as primary amino and sulfhydryl as well as tyrosyl. Since quinones are produced by tyrosinase from the phenolic activators, these quinones

[3] Suitable controls demonstrated that the final oxidation products of $FeCl_3$ and catechol did not inactivate invertase.

would be expected to react with all the above groups (12) to produce a typical tanning action on proteins (13, 14) in general, resulting in a complex in which the pigment is bound to the protein.

Considerable evidence has accumulated that many proteins form a pigment-protein complex produced during the oxidation of the catechol or dopa by tyrosinase (see (13)). Unfortunately, the protein concentration of invertase is too low for such studies, but with other proteins clear evidence was found for the formation of a protein-pigment complex. It was found that insulin, globulin, gelatin, serum albumin, and fibrinogen formed pigment-protein complexes when phenols were oxidized by tyrosinase in the presence of these proteins. The attachment of the pigment to the protein was followed by the formation of insoluble protein-pigment material which could easily be filtered from the solution when several soluble pro-
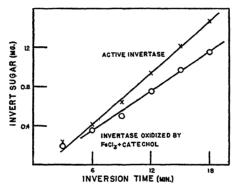
FIG. 4. Invertase activity is decreased by oxidation with FeCl₃ plus catechol. Catechol was omitted from the control.

teins were used as substrate. Also with certain very soluble proteins there could be formed suspensions of pigment-protein. The pigment could not be separated from the protein by adsorption on charcoal, filtration, or centrifugation. Nitrogen determinations on the solutions after the removal of the pigment-protein complex showed that the protein was fixed to the pigment. Since protein-pigment complexes were readily formed with a protein like gelatin, which contains almost no phenolic or sulfhydryl groups, it seems likely that these groups play only a secondary rôle in the inactivation of proteins by the tyrosinase-phenol system.

Since these solubility studies indicated that other proteins could be oxidized by tyrosinase plus phenolic activator, preliminary studies were made to see whether the biological activity of other proteins could also be impaired by oxidation by tyrosinase plus catechol.

Typical results on the effect of tyrosinase plus catechol on the hypo-

glycemic action of insulin are presented in Fig. 5. The insulin (25 mg.) had been oxidized at 37° at pH 6.0 with 0.5 ml. of Treemond tyrosinase plus 1 mg. of catechol for 4 hours. The control contained boiled tyrosinase, but was otherwise the same. The assay for insulin activity was made with rabbits in the usual way (United States Pharmacopoeia) and the blood sugar was followed for 6 hours (Fig. 5). The insulin activities after a
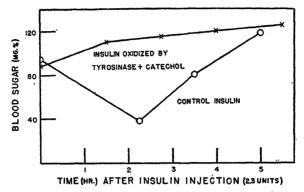
FIG. 5. The activity of insulin after oxidation by tyrosinase plus catechol is compared with a control sample in which boiled tyrosinase plus catechol was used. The same rabbit was used on different days for the assay.

TABLE II

Action of Tyrosinase Plus 1 Mg. of Catechol on Inactivation of Proteases

The proteases were assayed at optimum pH and at 37° by measuring the rate of digestion of a collagen filament (16).

Protease	Per cent inactivation
Pepsin	65
	32
Trypsin	76
	36
Chymotrypsin	48

typical experiment were also assayed on mice by the Armour Research Laboratories.[4] The results of this study showed an insulin activity of 6.7 ($\pm$13 per cent) units per mg. in the control and of 1.9 ($\pm$15 per cent) units per mg. in the insulin oxidized by tyrosinase plus catechol.

Preliminary experiments on pepsin, trypsin, and chymotrypsin indicated that these enzymes could be partially inactivated by tyrosinase plus

[4] It is a pleasure to acknowledge the aid of Dr. R. E. Thompson of Armour and Company in the assay of insulin.

catechol (see Table II), although previous work (1, 15) indicated that no inactivation was produced by tyrosinase alone.

DISCUSSION

The inactivation of biologically active proteins such as enzymes and hormones by tyrosinase plus a phenolic activator seems to be a fairly general phenomenon, since all proteins used in this study, including invertase, pepsin, trypsin, chymotrypsin, and insulin, were inactivated. Since the reaction does not seem to be specific for tyrosyl groups but probably involves amino, sulfhydryl, and other groups as well, it appears likely that most active proteins can be inactivated in this way. The possible significance of this reaction in vivo is much more difficult to ascertain at this stage of the investigation. It seems unlikely that such activators as catechol, cresol, or phenol would occur in tissues in sufficient amounts to be important in the tyrosinase system. On the other hand, since we have obtained some preliminary evidence for the presence in mushrooms of tyrosine or a tyrosine derivative, it seems quite possible that an activator similar to tyramine or epinine might play a rôle in the oxidation by tyrosinase of certain refractory substrates in vivo. It also must be kept in mind that other types of activators such as certain metal ions (unpublished work) might facilitate the oxidation of proteins by tyrosinase.

SUMMARY

Different tyrosinase preparations vary greatly with reference to their ability to inactivate invertase. The addition of very small amounts of certain phenolic compounds such as catechol, p-cresol, phenol, epinine, and tyramine, but not tyrosine or dopa, always resulted in very rapid and extensive inactivation of invertase by the tyrosinase. It appears that the phenolic activators owe their effectiveness to the fact that they are oxidized to quinone intermediates which are toxic to invertase. Protein inactivation by tyrosinase plus phenolic activator is not specific for invertase, since this oxidation system also inactivates pepsin, trypsin, chymotrypsin, and insulin and probably other biologically active proteins as well. The oxidation products of certain phenols combine with most proteins to form inert protein-pigment complexes.

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CONCENTRATION OF BOUND PANTOTHENIC ACID*

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The widespread existence in living tissue of combined pantothenic
acid unavailable to lactic acid bacteria has been recognized for some
time (1, 2), and two such bound forms, coenzyme A (3) and pantothenic
acid conjugate (4), have been extensively purified. The fractionation
was guided in these cases by following a specialized biological activity
possessed by the intact complex in each case.

The recent discovery of an enzymatic method of liberating pantothenic
acid from naturally occurring bound forms (5) offered a further method
for following the progress of isolation work. In the present study efforts
have been made to concentrate bound pantothenic acid, as measured
by differential *Lactobacillus arabinosus* assay, before and after suitable
enzyme treatment.

EXPERIMENTAL

Analytical Methods—Enzymatic liberation of pantothenic acid was car-
ried out as previously described (6), and the free or liberated vitamin
determined microbiologically by the method of Wright and Skeggs (7).
Acetylation (coenzyme A) activity was determined according to Kaplan
and Lipmann (8). Glutamic acid was measured after hydrolysis with
2 N acid by Henderson and Snell's method (9).

Enzymatically synthesized citrate was determined by Perlman's (10)
modification of Pucher's method (11), as improved by Natelson *et al.*
(12). The tubes containing the pentabromoacetone were chilled after
extraction and before addition of sodium sulfide. Pantoic acid was liber-
ated by hydrolyzing the sample in 2 N sulfuric acid for 2 hours at 120°

and subsequently opening the lactone ring with 2 N sodium hydroxide at 120° for 15 to 30 minutes. The liberated pantoic acid was then determined with *Acetobacter suboxydans* according to the method of King and Cheldelin (13). β-Alanine, likewise released by hydrolysis with 2 N acid, was measured with *Saccharomyces carlsbergensis* (14). Adenine was calculated from the optical density at 260 mμ measured with a Beckman spectrophotometer. The usual Fiske and Subbarow procedure (15) was used for phosphorus, total phosphate being determined after sulfuric acid-hydrogen peroxide treatment.

Fractionation of Boiled Extracts—Liver and yeast are among the best available sources of pantothenic acid, and, if fresh, contain practically all of the vitamin in a bound (*i.e.*, microbiologically unavailable) form. They were, therefore, selected as source materials at the start of this work.

Beef liver was cut into slices, chilled with chipped ice, passed through a meat grinder, mixed with 1 liter of boiling water per kilo, and the mixture was steamed for 10 minutes. This process was completed within 40 to 60 minutes after the death of the animal. The extraction was repeated four times, the supernatant being decanted each time.

Actively fermenting brewers' yeast was drained from a commercial fermenter,[1] the cells were collected by suction filtration, mixed with 3 liters of boiling water per kilo (fresh weight), and the mixture was steamed 20 minutes. The cells were allowed to settle, the supernatant decanted, and the extraction repeated twice.

The filtered extracts containing essentially all of the original pantothenic acid activity, 90 to 99 per cent in the bound form, were concentrated *in vacuo* to about 500 ml. per kilo of starting material, and alcohol was added to 70 per cent concentration. The precipitate had to be redissolved and reprecipitated four or five times to remove all of the activity. The alcoholic solutions were then concentrated to a syrup *in vacuo* and taken up in about 200 ml. of water per kilo of original weight.

Such concentrates were used for investigating the possibility of adsorbing or precipitating the desired material. Treatment of the neutral extracts with animal or plant charcoals, calcium carbonate, magnesium oxide, fullers' earth, or aluminum hydroxide Cγ (16), in amounts equal to 10 per cent of the dry weight of the extract, did not remove significant amounts of bound pantothenic acid.[2] Also, the activity was not completely

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[1] Fresh brewers' yeast was very kindly furnished by the Fauerbach Brewing Company, Madison.

[2] LePage and Mueller (24) have perfected a method for isolation of codehydrogenases in which the activity is adsorbed from a crude liver extract with charcoal. Small aliquots of one of these extracts before and after charcoal treatment were

precipitated at neutral pH with excess Ag^+, Hg^+, Ba^{++}, or the cuprous chloride reagent of Jandorf (17).

However, fractional precipitation with basic lead acetate, sodium hydroxide being added as required to maintain approximate neutrality, showed some promise. When this reagent was used in successive portions equal to 6, 15, and 100 per cent of the dry weight of the extract, respectively, nearly all of the activity was found in the third precipitate. Any free pantothenic acid present, usually 1 to 5 per cent of the total, remained in the filtrates.

Such concentrates, after removal of lead as the sulfide, contained about 35 mg. of bound pantothenic acid per kilo of liver, or 15 mg. per kilo of yeast. The purity of each preparation was approximately 0.1 per cent, as pantothenic acid, on a dry weight basis.

More recently boiling water extracts of fresh rat liver have been deproteinized either with trichloroacetic acid (10 per cent final concentration) or by concentrating the extracts to a small volume *in vacuo* and extracting repeatedly with liquid phenol. The extracts obtained by either method contained most of the original bound pantothenic acid in a purity approximately equal to that obtained from the basic lead acetate fractionation of the boiled yeast or liver extracts.

Fractionation of Cyclophorase Preparations—At this stage of the work the barium-insoluble fraction of the trichloroacetic acid (TCA) filtrate from a rabbit liver cyclophorase preparation[3] was found to contain nearly 0.5 per cent of bound pantothenic acid on the dry weight basis. It, therefore, appeared that a large portion of the bound pantothenic acid in fresh liver homogenates must be rather firmly attached to the cell nuclei or mitochondria contained in the cyclophorase gel and that the removal of lower molecular weight, water-soluble materials in the supernatant from the centrifuged gel had already resulted in a considerable concentration. For these reasons attention was turned to cyclophorase as the starting material for further fractionation.

The liver was removed from three freshly killed rabbits and found to weigh 255 gm. Each liver was at once homogenized for 1 minute with 250 ml. of ice-cold 0.9 per cent potassium chloride solution in a cold Waring blendor. The homogenates were centrifuged for 5 minutes at

kindly made available for analysis. In this case 73 per cent of the bound pantothenic acid was found in the charcoal filtrate. Later a sample of the purified Fraction 2 obtained from cyclophorase (see below) was shaken with norit, and all the yellow color, coenzyme A activity, and bound pantothenic acid were adsorbed. However, elution proved to be difficult, and the use of charcoal was not further investigated.

[3] Kindly supplied by Dr. David Green.

1765 × g and 3° in a refrigerated centrifuge. The supernatants were decanted and the gels washed back into the blendor with sufficient of the above saline to make a total volume of 250 ml. While the blendor was running, 25 ml. of TCA solution containing 1 gm. per ml. was gradually added, and the homogenization continued for 30 seconds longer. The elapsed time from the death of the animals to this stage was 24 minutes.

The precipitate was centrifuged as previously described, the yellow supernatants decanted, and the precipitate rehomogenized with 250 ml. of 5 per cent TCA solution. After centrifuging, the combined supernatants were extracted in a separatory funnel three times with equal volumes of ether to remove most of the TCA, and the aqueous layer was then adjusted to neutrality with dilute sodium hydroxide. In this run the TCA extract contained about 12 per cent of the bound pantothenic acid of the original liver.

To date fourteen cyclophorase preparations have been made by the above procedure or minor modifications thereof. Beef, hog, and sheep livers have been used, as well as rabbit liver. Several runs, starting with 400 to 1500 gm. of beef or hog liver, were put through a Sharples supercentrifuge, in the hope that more of the particulate matter of the liver homogenates would be sedimented in the stronger centrifugal fields produced (about 30,000 × g). A 405 gm. portion of beef liver containing 18.9 mg. of bound pantothenic acid, when treated in this manner, yielded 7.68 mg. (40 per cent) in the TCA extract. The results on larger batches were less satisfactory. Every effort has been made to minimize autolysis by working as quickly as possible and keeping the material at a temperature close to freezing. The elapsed time from death of the animal to addition of TCA has varied from 24 to 150 minutes in the runs made.

In spite of these precautions, the yield of bound pantothenic acid in the TCA filtrates has been erratic and frequently very low, ranging from about 3.5 to 40 per cent. Analysis of the cell nuclei, mitochondria, and microsome fractions of a single rat liver, which had been perfused with cold 0.9 per cent potassium chloride solution immediately after death,[4] showed a yield of 70 per cent of the original bound pantothenic acid in the three combined fractions. Perfusion of larger animal livers was, therefore, carried out, but the yields were not improved over the 3.5 to 40 per cent range previously obtained.

Barium Precipitation—It was found that considerable purification of the TCA filtrates could be achieved through barium precipitation. The procedure was carried out as follows.

The neutralized TCA extract obtained from liver from three rabbits

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[4] Prepared by Dr. J. M. Price, McArdle Memorial Laboratory, Medical School, University of Wisconsin.

as described above was concentrated *in vacuo* at 50° to 40 ml. The solution was cloudy but could not be cleared by filtering or centrifuging, although in other runs it was found that much of the solid would settle out on standing overnight at 0–5°. The concentrated extract was treated with 50 ml. of 25 per cent barium acetate solution, 400 ml. of alcohol, and sufficient dilute sodium hydroxide to give a faint pink color with phenolphthalein. The mixture was held 12 hours at 0–5°, centrifuged, and the precipitate dissolved in sufficient dilute hydrochloric acid to give pH 5.0. The solution was rechilled, dilute sulfuric acid cautiously added until the pH reached 1.5, and the barium sulfate centrifuged and washed

TABLE I

*Distribution of Bound Pantothenic Acid in Various Solvent Systems**

Solvent phase	Aqueous phase	pH of aqueous phase†	Distribution ratio‡
2,4,6-Collidine	0.005 M KCl	7.0	25
n-Butanol	0.001 " acetic acid	5.0	40
"	Water	7.0	47
o-Cresol	"	7.0	17
Phenol	10% ammonium acetate	7.0	15
"	0.1 M " "	4.0	3.9
19:1 phenol-chloroform	0.1% KCl	7.0	8
Phenol	0.05% "	3.0	1.7

* A concentrate containing 20 to 40 γ of bound pantothenic acid was dissolved in the aqueous phase and equilibrated with an equal volume of the organic solvent in a separatory funnel. Each layer was freed of organic solvent by ether extraction (water added as necessary) before analysis.

† pH of aqueous phase adjusted before equilibration.

‡ Ratio of bound pantothenic acid in the water phase to that in the organic solvent phase.

twice with dilute sulfuric acid of the same pH. The combined supernatants were neutralized and stored in the frozen state.

Such preparations contained 75 to 90 per cent of the pantothenic acid present in the TCA extracts and were of 0.2 to 0.6 per cent purity on the dry basis. No free pantothenic acid was present.

Solvent Distribution Studies—A search was made for a water-immiscible solvent in which the bound pantothenic acid would be sufficiently soluble to permit fractionation according to Craig's procedure (18). Preliminary single bulb distributions (Table I) indicated that of the solvents tried only phenol extracted an appreciable fraction of the bound pantothenic acid from aqueous solutions, and that extraction was favored by acidifying the aqueous phase. Formation of emulsions was rather trouble-

some but was partially prevented by the presence of salt in the aqueous phase. On the basis of these observations concentrates prepared as above were distributed in a phenol-water system as described below.

An aqueous 0.05 per cent potassium chloride solution was acidified to pH 3.0 with hydrochloric acid and equilibrated with phenol by vigorous shaking in a large separatory funnel. A series of twelve, 250 ml. separatory funnels was mounted on a wooden rack, and 100 ml. portions of the clear upper (aqueous) phase added to each funnel except the first.

A solution prepared from 400 gm. of rabbit liver by the cyclophorase-barium fractionation was adjusted to pH 3.0, diluted to 100 ml., and placed in Bulb 1. An equal volume of the equilibrated phenol phase was then added, and the funnel was shaken or swirled for about 1 to 2 minutes. After the layers had separated, which sometimes required centrifuging in the case of the first few bulbs in the series, the lower (phenol) layer was transferred to Bulb 2, fresh phenol phase added to Bulb 1, and the distribution completed according to the general procedure of Craig (18).

The aqueous phase from each funnel was then extracted three or more times with 0.33 volume of ether until the phenol concentration was reduced to approximately 1 part in 100,000, as determined by Ettinger's (19) use of Gibb's reagent, and was then neutralized and concentrated *in vacuo* to remove dissolved ether. Each phenol phase was diluted with 2 to 3 volumes of ether and extracted with five successive 15 to 20 ml. portions of water. The combined water extracts, which contained all of the activity, were then freed from phenol and ether as above and neutralized. Each sample was assayed for total pantothenic acid after the double enzyme treatment. No free pantothenic acid was detectable in any of the Craig fractions. The results are plotted in Fig. 1.

A total of eleven such distributions has been carried out by using as starting material concentrates prepared from rabbit, beef, and hog liver cyclophorase, and from the phenol-soluble fraction of aqueous rat liver extracts. In all cases, as in Fig. 1, two distinct subfractions containing bound pantothenic acid were found to have been partially separated by the distribution process, although the exact shape of the curves and the location of the main peak (Bulb 4, Fig. 1) varied somewhat in the different runs. The main fraction, appearing mostly in Bulbs 2 to 6, was relatively more soluble in the aqueous than in the phenol phase, whereas the second fraction, which was present in increasing amounts in Bulbs 7 to 12, was much more soluble in phenol. After completing the distribution, the contents of Bulbs 2 to 6, comprising the main peak, were combined to form a composite sample of the more water-soluble component and designated Fraction 1. Similarly the more phenol-soluble component was collected from Bulbs 7 to 12 and designated Fraction 2.

In an effort to effect a more clear cut separation, preparations of the two fractions were separately redistributed in the same solvent system. The results are given in Fig. 2. The shape of the curves obtained from Fraction 1 suggests that this sample still contained some of the phenol-soluble material, or perhaps was giving rise to it during the distribution. Fraction 2, however, gave no evidence of containing a second pantothenic acid-containing component.
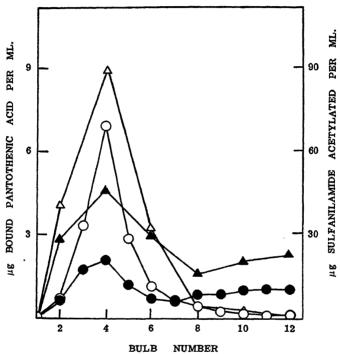
FIG. 1. Distribution of bound pantothenic acid and coenzyme A activity in a phenol *versus* 0.05 per cent aqueous KCl system.   O, pantothenic acid content, water phase; ●, pantothenic acid content, phenol phase; △, coenzyme A activity, water phase; ▲, coenzyme A activity, phenol phase.

Properties of Fractions 1 and 2—Preparations of the two fractions obtained both from cyclophorase and from hot water extracts of liver are listed in Table II. The samples of Fraction 1 were perfectly clear, colorless solutions, while the others though clear were distinctly yellow. The most highly purified samples (from cyclophorase) contained about 4 to 5 per cent of bound pantothenic acid on the basis of total organic solids present. These figures undoubtedly correspond to a several fold higher

percentage of the actual bound forms. In certain cases the pantothenic acid content was checked by determinations of pantoic acid after hydrolysis. Reasonably good agreement was found (Table II). Similar results based on β-alanine determinations (not shown in Table II) were about 20 per cent high.

The more concentrated samples also contained glutamic acid and phosphorus, the latter being somewhat lower in the phenol-soluble fraction.
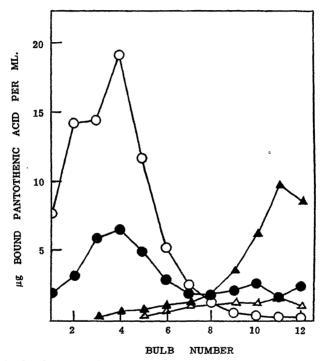
FIG. 2. Redistribution of Fractions 1 and 2 (see the text) in the solvent system of Fig. 1. O, water phase from redistribution of Fraction 1; ●, phenol phase from redistribution of Fraction 1; △, water phase from redistribution of Fraction 2; ▲, phenol phase from redistribution of Fraction 2.

The same components were also present in the fractions obtained from aqueous extracts, but have little interest because of the low purity of these preparations (Table II).

Both fractions were found active in catalyzing the acetylation of sulfanilamide. Difficulty in estimating this activity quantitatively was experienced in that the amount acetylated by a given concentrate varied by a factor of several fold on successive trials. The chief source of this

variation appeared to lie in the apoenzyme preparation used. However, by comparing only results secured in a single series of analyses with a given set of reagents, a relative indication of acetylating activity was obtained. In this manner the individual samples from the Craig series plotted in Fig. 1 were tested, and the acetylation activity was found to parallel the bound pantothenic acid content. These results are also shown in Fig. 1.

TABLE II

Properties and Composition of Two Bound Pantothenic Acid Fractions

Property or component	Preparations from cyclophorase		Preparations from boiling water extracts of liver	
	Fraction 1	Fraction 2	Fraction 1	Fraction 2
	Micrograms per ml.			
Total organic solids.....................	600	72	14,500	18,650
Bound pantothenic acid, *L. arabinosus**.	23.2	3.6	33.0	17.8
" " " *A. suboxydans*†.			27.3	20.7
	Moles per mole pantothenic acid‡			
Total phosphorus......................	23	9.3	49	48
" glutamic acid...................	1.3	1.4	20	28
Flavin-adenine dinucleotide............	0	1.4		
Adenine...............................			2.0	6.8
	Acetylation activity, moles formed per mole pantothenic acid			
Acetyl sulfanilamide§...................	10.8	28.3	29.2	50.5
Citric acid‖............................			7.7	11.7

   * Determined after digestion with intestinal phosphatase and chicken liver enzyme.
   † Pantothenic acid calculated from pantoic acid found after hydrolysis.
   ‡ Based on values from *L. arabinosus* assay.
   § In 2 hours.
   ‖ In 1 hour.

Although somewhat obscured by the analytical variability mentioned above, a tendency for Fraction 2 to show greater acetylation activity than Fraction 1 per unit weight of pantothenic acid present was noted. Illustrative data are included in Table II. The activity of the two fractions from cyclophorase was determined at one time and that of the boiled extract preparations at another.

   Both fractions from the aqueous liver extract were effective in catalyzing the formation of citric acid from acetate and oxalacetate when

an aged, dialyzed extract of acetone powder from pigeon liver was used as the apoenzyme (Table II). The cyclophorase fractions have not been tested for activity in citrate synthesis but presumably are active. The degree of activity per unit weight of pantothenic acid was about one-eighth of that found by Stern and Ochoa (20). The difference is again probably attributable to the apoenzyme preparation used.

In view of the observation that certain naturally occurring forms of pantothenic acid are more resistant to alkali than the free vitamin (2, 4, 21), it was of interest to test the alkali stability of concentrates prepared in the present work. Preparations such as those listed in Table II were made 1 N with respect to sodium hydroxide and autoclaved at 15 pounds pressure (120°) for 90 minutes. Under these conditions the acetylating ·power was rapidly and completely lost, as was to be expected from previous work (22). The neutralized solutions contained no free pantothenic acid, but, when subjected to the routine double enzyme treatment, gave rise to a considerable fraction of the original activity, which varied in an unexplainably erratic manner from around 10 to nearly 100 per cent. In no case was all the activity destroyed by alkali treatment. No consistent difference in the behavior of Fractions 1 and 2 in this regard could be established.

DISCUSSION

Throughout this work it has been noted that the pantothenic acid of fresh tissue such as liver exists almost entirely in a bound state, but is very rapidly converted into the free form on autolysis. However, after enzyme-inactivating treatments, this tendency is entirely eliminated, and the bound form or forms then possess ample stability for ordinary chemical fractionation procedures. Any free pantothenic acid present in the source material or arising from partial autolysis may be separated readily from the bound vitamin, for example, by Pb^{++} or Ba^{++} precipitation as described in the "Experimental."

The preparation of concentrates of bound pantothenic acid via cyclophorase is a procedure best adapted to rather small scale operation. Application to quantities of more than a pound or two of liver appears impractical at present. Furthermore, as emphasized above, the yields obtained are variable and seldom over 40 per cent. However, in spite of these difficulties, the procedure outlined appears to be the best method available at present for obtaining small amounts of rather highly purified concentrates.

The results of the Craig separations establish the existence in the materials studied of two different substances containing bound pantothenic acid and possessing the typical catalytic activity of coenzyme A. It seems probable that the two active substances present are closely related

chemically.   In this connection it is of interest that Cheldelin, Teply, and Green have recently obtained bound pantothenic acid concentrates from cyclophorase, which on distribution in the phenol-water system used in the present work show practically no movement of the activity out of Bulb 1 of the series.[5]

Whether the two forms exist in the original tissue or are produced during fractionation by partial hydrolysis of some more complex molecule cannot be decided on the basis of present information.   It would seem unlikely that breakdown products would still retain coenzymic activity, unless the degradation involved was slight and of an easily reversed nature. This could conceivably be the case if the various forms differed only in degree of phosphorylation, since adenosine triphosphate was present in all the coenzyme test systems.

The behavior of the bound forms during the fractionation procedures used in this work emphasizes their acidic nature.   The presence of an acid grouping is indicated by the precipitation with metals and by the effect of lowered pH on the solubility in non-aqueous solvents (Table I).   Preliminary experiments with ion exchange resins point in the same direction. Only a small part of the activity was held on a cation column, whereas all was retained by an anion exchanger and could be eluted with dilute aqueous ammonia solutions.

The partial stability of bound pantothenic acid noted in the present work is in marked contrast to the alkali sensitivity of the free vitamin (23).   Evidently the additional constituents are attached in such a way as to stabilize the amide linkage.   What the point of attachment may be is a matter of conjecture at present.

SUMMARY

Differential assays of free and total pantothenic acid with *Lactobacillus arabinosus*, before and after suitable enzyme treatment, have been used as a guide in efforts to concentrate the bound forms from yeast and liver. Concentrates obtained both from hot water extracts of liver and from liver cyclophorase were separated into two fractions by means of solvent distribution in a phenol-water system.   Each fraction contained a different bound form of pantothenic acid, both of which possessed typical coenzyme A activity.   The most highly purified concentrates, obtained via cyclophorase, contained 4 to 5 per cent of pantothenic acid, all in the bound form.

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A COLOR TEST FOR FRUCTOSE

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The ketose most commonly found in biological material is fructose. The reaction ordinarily used for the identification of this ketose is the well known Seliwanoff test which depends on the formation of 4-hydroxymethylfurfural and its reaction with resorcinol to form a red-colored compound.

Niederl and Maurmeyer in their study of sugar-phenol condensations[1] observed that a reddish brown color was produced when glucose was condensed with phenol under specified experimental conditions.

It was decided to study the usefulness of this technique for the identification, by way of color reactions, for other sugars. The interesting observation was made that of all the sugars examined fructose alone gave a green color when treated according to the experimental procedure described below.

A stock solution of fructose was prepared by dissolving 0.5 gm. in 100 ml. of glacial acetic acid; the solution of phenol was made by dissolving 0.25 gm. in 100 ml. of glacial acetic acid. 10 drops of this phenol solution were mixed with 3 drops of concentrated sulfuric acid and 4 drops of the fructose solution. The color was then developed by placing the test-tube in boiling water for a period of at least 5 minutes. A longer period of heating, not exceeding 15 minutes, leads to the formation of a more intense green color. Periods of heating in excess of 15 minutes caused the disappearance of the green color, the latter being replaced by a dark brown.

A distinct green color is still produced by 0.1 mg. of fructose in 1 ml. of solution. Low water, low sulfuric acid, and high phenol concentrations appear to favor the production of the green color.

The procedure was applied to numerous sugars and the results obtained are indicated in Table I. An examination of Table I reveals that in addition to fructose those substances yielding fructose on hydrolysis also give a green color.

In order to determine whether the green color produced due to the presence of fructose is observable in the presence of larger concentrations of other sugars, a mixture of 1 mg. of fructose and 50 mg. of glucose was subjected to the above procedure. A distinct green color was noted.

At the suggestion of Dr. Louis Sattler, Brooklyn College, a sample of

[1] Niederl, J. B., and Maurmeyer, R. K., *J. Am. Chem. Soc.*, **61,** 1005 (1939).

hydroxymethylfurfural, kindly supplied by him, was tested and a distinct green color developed. It appears, therefore, that the test, as in the case of the Seliwanoff reaction, is due to this substance. It is felt, however, that the procedure described here is unique in that it states experimental conditions which readily lead to the formation of a green color in the presence of fructose. The test can conceivably be used as a confirmatory test in conjunction with the Seliwanoff reaction.

TABLE I

Results of Color Test with Various Sugars

Substance tested	Color developed
Arabinose	Brown
Fructose	Green
Galactose	Brown
Glucose	Yellow
Glycogen	"
Inositol	"
Inulin	Green
Lactose	Yellow
Mannose	Red
Raffinose	Green
Rhamnose	Red
Sucrose	Green
Xylose	Pink

SUMMARY

Fructose or products yielding fructose under the stated experimental conditions will yield a green color in the presence of phenol.

It appears that the color produced is due to the interaction of hydroxymethylfurfural and phenol.

CHOLESTEROL ESTERASES

III. OCCURRENCE AND CHARACTERISTICS OF CHOLESTEROL ESTERASE OF SERUM*

By LEON SWELL AND C. R. TREADWELL

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Sperry and Brand (1) have discussed the possible importance of cholesterol esterase in the absorption and metabolism of cholesterol. While earlier work had suggested that whole blood had cholesterol esterase activity, Sperry (2) was the first to report esterifying activity in serum. In a series of studies (1-4), Sperry and coworkers reported that esterification of the free cholesterol occurred when human or dog serum was incubated alone, that the reaction was absent in serum heated for 1 hour at 55-60°, and that the optimum pH for the reaction was about 8. It was found that bile salts had a marked effect on this esterification reaction. In human serum, as the bile salt concentration was increased, the esterification decreased, and, above a certain concentration of bile salts, there was neither esterification nor hydrolysis. With dog serum there was a decrease in esterification as the bile salt concentration was increased until a point was reached at which no esterification or hydrolysis occurred. With amounts of bile salts above this level the cholesterol esters of dog serum were hydrolyzed more or less in proportion to the concentration of bile salt until with large amounts complete splitting had occurred.

Two recent reports (5, 6) have described the preparation of substrate mixtures, suitable for studying the hydrolyzing and esterifying cholesterol esterase systems, and the employment of these mixtures in the characterization of the enzyme in pancreatin. The present report gives the results of an investigation of the occurrence of cholesterol esterase in the serum of five species, namely, human, dog, rat, rabbit, and guinea pig. When these were tested according to our procedures (5, 6), only dog serum exhibited cholesterol esterase activity. Further experiments demonstrated that the enzyme in dog serum was like that previously characterized in pancreatin (5, 6). Inasmuch as our results did not confirm the reports of Sperry and coworkers in respect to the cholesterol esterase activity of human serum, it seemed desirable to test human and dog sera under conditions closely simulating those employed in the earlier studies. These

experiments confirmed the earlier findings that the free cholesterol of human and dog sera was esterified during incubation. However, as will be discussed below, this esterification reaction is distinctly different from the cholesterol esterase activity in dog serum which was demonstrated by our procedures.

<div align="center">EXPERIMENTAL</div>

Preparation of Enzyme Digests and Determination of Activity in Serum— Various amounts of serum were added to emulsions of cholesterol oleate, or of cholesterol and oleic acid, prepared as described previously (5, 6) and incubated under various conditions (Tables I, II, and III). The volume of phosphate buffer was reduced by the amount of serum added (up to 4 cc.); thus the total volume incubated was always 13 cc.

*Experiments on Incubation of Human and Dog Sera—*The effect of incubation on the free cholesterol of human and dog sera was tested under the following conditions. First, 3 cc. of serum, to which 0.1 cc. of 0.001 per cent merthiolate had been added, were incubated either alone or with the addition of 0.5 cc. of 10 per cent sodium taurocholate (2, 4). Secondly, 3 cc. of serum plus 2 cc. of emulsions (5, 6) of either cholesterol oleate or cholesterol and oleic acid were incubated. The composition of the emulsions was varied as indicated in Table IV. Samples were removed for analysis at 0, 6, 24, 48, 72, and 96 hours of incubation.

*Serum—*The human serum used in any single test was from a pooled sample obtained from two or more donors during the forenoon of the day the experiment was started. The serum of the other species was obtained from animals maintained on commercial laboratory chow and not undergoing any other treatment. The serum used in the experiments shown in Tables II and III had been stored overnight in the refrigerator. In the other experiments the serum was obtained during the forenoon of the day it was used.

*Determination of Cholesterol and Calculation of Results—*The total and free cholesterol of the samples was determined by the method of Schoenheimer and Sperry (7) as modified by Sperry (8). The total cholesterol was determined in all zero hour samples and in the later samples when it was necessary to check the uniformity and stability of the emulsions during incubation. All calculations were based on the amounts of cholesterol found on analysis to be present in the digests before incubation was started. The data obtained with dog serum and our previously described procedures (5, 6) are given in Tables I, II, and III as the per cent hydrolysis or esterification. In Table IV the results of the experiments on the incubation of human and dog sera are expressed as the free cholesterol content after various periods of incubation.

Results

Cholesterol Esterase Activity of Serum in Five Species—Enzyme digests (see above), containing 1 or 2 cc. of serum, were assayed for activity in the presence and absence of bile salts and at several pH levels from 5.9 to 8.3. No significant cholesterol esterase activity was found in human, rabbit, rat, or guinea pig serum. However, dog serum contained highly active esterifying and hydrolyzing cholesterol esterase systems, which could be demonstrated in the presence, but not in the absence of bile salts. Additional studies were then made with dog serum to characterize

TABLE I

Influence of pH on Hydrolyzing and Esterifying Cholesterol Esterase Systems of Dog Serum

The enzyme digests were prepared as described in the text. Substrate for hydrolysis, cholesterol oleate, 75 mg.; for esterification, cholesterol, 25 mg., oleic acid 54.8 mg., sodium taurocholate, 100 mg. Enzyme, 3 cc. of dog serum.

Digest No.	pH	Hydrolysis		Digest No.	pH	Esterification	
		6 hrs.	24 hrs.			6 hrs.	24 hrs.
		per cent	*per cent*			*per cent*	*per cent*
1	5.7	7.3	8.1	10	5.9	12.1	42.2
2	5.9	12.2	18.7	11	6.1	13.9	47.9
3	6.3	12.6	20.9	12	6.3	10.4	35.8
4	6.4	13.4	22.8	13	6.5	10.9	32.1
5	6.7	10.6	19.4	14	6.8	0.7	0.0
6	6.8	9.1	10.9	15	7.3	0.0	0.0
7	7.3	7.6	8.6				
8	7.5	8.1	8.3				
9	8.9	6.6	6.8				

the enzyme. The results of these studies are summarized in the two following sections.

Influence of pH on Hydrolyzing and Esterifying Cholesterol Esterase Systems of Dog Serum—The data in Table I show that the optimum pH for esterification was 6.1, essentially the same as was found for the enzyme in pancreatin (6). It will be noted that in dog serum, as in pancreatin, the esterifying activity disappeared at about pH 6.7. The optimum pH for hydrolysis was approximately the same as that found with pancreatin (5). The data also demonstrate the wide pH range of the hydrolytic system.

Influence of Bile Salt and Serum Concentration on Hydrolysis and Synthesis of Cholesterol Esters by Dog Serum—The results are shown in Tables II and III. Dog serum showed no activity, either hydrolytic or esterify-

ing, in the absence of bile salts when tested according to our routine procedures.   Hydrolysis and esterification were increased with increasing concentrations of sodium taurocholate.   After 6 hours incubation the ac-

TABLE II

Influence of Bile Salt Concentration on Hydrolyzing and Esterifying Cholesterol Esterase Systems of Dog Serum

The enzyme digests were prepared as described in the text.   Substrate for hydrolysis, cholesterol oleate, 75 mg.; for esterification, cholesterol, 25 mg., oleic acid, 54.8 mg.   Bile salt solution for hydrolysis, 1 cc. of 10 per cent Tween 20 containing sodium taurocholate; for esterification, 1 cc. of sodium taurocholate solution.   pH of digests for hydrolysis, 6.6; for esterification, 6.2.   Enzyme, 3 cc. of dog serum.

Sodium taurocholate	Hydrolysis		Esterification	
	6 hrs.	24 hrs.	6 hrs.	24 hrs.
mg.	per cent	per cent	per cent	per cent
· 0	0.0	0.0	0.0	0.0
10	0.0	1.5	0.8	8.6
30	4.0	8.3	3.3	19.6
50	5.2	9.0	5.6	17.7
100	10.5	13.2	12.7	20.6

TABLE III

Effects of Different Amounts of Dog Serum on Synthesis and Hydrolysis of Cholesterol Esters

The enzyme digests were prepared as described in the text.   Substrate for hydrolysis, cholesterol oleate, 75 mg.; for esterification, cholesterol, 25 mg., oleic acid, 54.8 mg., sodium taurocholate, 100 mg.   pH of digests for hydrolysis, 6.6; for esterification, 6.2.

Serum	Hydrolysis		Esterification	
	6 hrs.	24 hrs.	6 hrs.	24 hrs.
cc.	per cent	per cent	per cent	per cent
3*	0.0	0.0	0.0	0.0
0	0.0	0.0	0.0	0.0
1	0.5	5.0	3.6	8.0
2	4.9	7.9	7.4	11.2
3	5.3	10.8	10.0	13.9
4	10.0	12.9	11.1	18.1

* Heated at 65° for 15 minutes.

tivities were roughly proportional to the bile salt concentration.   The enzyme in dog serum was inactivated by heating at 65° for 15 minutes. The degree of hydrolysis and esterification was roughly proportional to the serum concentration after 6 hours incubation.

Esterification of Free Cholesterol of Human and Dog Sera during Incubation—The data shown in Table IV are representative of a large number of experiments on the effect of incubation on the free cholesterol content of human and dog sera. These experiments, which closely simulated the conditions and procedures employed by Sperry and coworkers, were carried out to obtain information which might resolve the apparent disagreement between the previously reported occurrence of cholesterol esterase activity in human serum and our negative findings in this regard.

TABLE IV

Free Cholesterol Content of Human and Dog Sera during Incubation

3 cc. of serum were used in all experiments. Substrate in emulsions for hydrolysis, cholesterol oleate, 12.5 mg.; for esterification, cholesterol, 6.5 mg., oleic acid, 9.2 mg.

Experiment No.	Emulsions	Substrate	Sodium taurocholate	pH	Free cholesterol			
					0 hrs.	6 hrs.	24 hrs.	48 hrs.
	cc.				*mg.*	*mg.*	*mg.*	*mg.*

Human serum

Experiment No.	Emulsions	Substrate	Sodium taurocholate	pH	0 hrs.	6 hrs.	24 hrs.	48 hrs.
1	0		−	7.5	2.7	2.3	2.3	2.2
2	0		+	7.5	2.8	2.7	2.7	2.7
3	2	Cholesterol, oleic acid	−	6.7	7.6	7.2	7.3	7.1
4	2	" " "	+	6.8	7.6	7.4	7.5	7.5
5	2	" oleate	−	7.0	3.2	2.6	2.6	2.6
6	2	" "	+	7.0	3.6	3.6	3.5	3.4

Dog serum

Experiment No.	Emulsions	Substrate	Sodium taurocholate	pH	0 hrs.	6 hrs.	24 hrs.	48 hrs.
7	0		−	7.6	2.3	1.5	1.6	1.6
8	0		+	7.7	2.3	6.2	6.5	6.3
9	2	Cholesterol, oleic acid	−	6.8	7.2	6.7	6.6	6.5
10	2	" " "	+	6.8	7.6	7.0	9.8	10.9
11	2	" oleate	−	6.9	2.8	2.4	2.4	2.5
12	2	" "	+	7.0	3.8	6.8	7.0	8.8

There were no significant changes in the free cholesterol values after 48 hours, and these have been omitted from Table IV. The results confirm the findings of Sperry and coworkers that esterification of the free cholesterol occurs during incubation of both kinds of serum and that the reaction occurs when the pH is below 8. The data show that the amount of cholesterol esterified was not influenced by the concentration of free cholesterol or of cholesterol ester, approximately 0.5 mg. being esterified in all cases (see Experiments 1, 3, and 5, Table IV). There was no evidence of hydrolysis in human serum even when the concentration of cholesterol ester was increased (Experiments 5 and 6). The results with dog

serum differed from those on human serum in that incubation of dog serum in the presence of bile salts produced hydrolysis. In Experiments 8 and 10 there was an increase in the free cholesterol equivalent to nearly 100 per cent of the esters present in the serum. In Experiment 12, in which additional cholesterol ester was present, the amount of ester hydrolyzed was still larger.

DISCUSSION

The present study demonstrated that dog serum contained an enzyme which, in the presence of bile salts, catalyzed the hydrolysis and synthesis of cholesterol esters. In respect to optimum pH, inactivation temperature, and the requirement for bile salts, this enzyme resembled the cholesterol esterase which we have previously characterized in pancreatin (5, 6). The absence of this enzyme in the serum of the human, rat, rabbit, and guinea pig suggests that the dog is unique as far as the occurrence of this enzyme is concerned. The presence of this enzyme in dog serum explains the different effects of bile salts in human and dog sera, as observed by Sperry and Stoyanoff (3). The addition of bile salts inhibited the esterification process which occurred in both sera during incubation but in dog serum activated the pancreatic type of enzyme.

The esterification reaction observed in the experiments on the incubation of serum (Table IV) seems to be identical with that studied by Sperry and coworkers and considered to be catalyzed by cholesterol esterase. In our experiments the incubation of serum resulted in the esterification of 20 to 30 per cent of the free cholesterol of the serum, whereas in the earlier studies the esterification ranged from 30 to 60 per cent. This lack of quantitative agreement was probably due to differences in experimental technique. Sperry reported that the esterification reaction did not occur when serum was diluted more than 1 to 5 cc. or heated for 1 hour at 60°. Our results show that the amount of cholesterol esterified, when human or dog serum was incubated, was quite constant and was not influenced by the concentration of substrate or end-product. We would point out that the evidence for the enzymatic nature of this reaction is not entirely convincing. The absence of esterification in heated serum may have been due to a change in the particular dispersion system of cholesterol in serum rather than inactivation of an enzyme. Also the absence of a dilution effect (up to 1 to 5 cc.) suggests that the reaction may not be enzymatic. Finally, the observation that added substrate or end-product did not influence the amount of cholesterol esterified may indicate that the reaction involves only free cholesterol dispersed as it occurs in serum. Thus, from the data of Sperry and of the present report, we suggest that the esterification reaction in incubated serum is either catalyzed by an enzyme of

very low activity or is non-enzymatic. Turner and Pratt (9) recently reported that the esterification of cholesterol in incubated serum was decreased in samples obtained from patients with disease of the liver parenchyma. Studies on the cholesterol esterase of liver, now in progress in our laboratory, may give information regarding the nature of this esterification reaction. Sperry and Brand (1) have pointed out that "Except for the hydrolysis of cholesterol esters in dog serum in the presence of bile salts reactions catalyzed by cholesterol esterases proceed at a very slow rate," and "If cholesterol took such an active part in the transport of fatty acids as is suggested by Schramm and Wolff [(10)], one might expect to find more active cholesterol esterases in animal tissues." It seems very probable that the slow reactions are distinctly different from the reactions catalyzed by the pancreatic type of cholesterol esterase. In most cases it is difficult to decide which of these reactions was studied by earlier workers. However, it seems likely that if a cholesterol esterase has an active part in the absorption and metabolism of cholesterol it is the highly active one characterized in our studies on pancreatin and dog serum.

SUMMARY

The serum of five species has been examined for cholesterol esterase activity. In dog serum, but not in human, rat, rabbit, or guinea pig serum, highly active hydrolytic and esterifying cholesterol esterase systems were demonstrated. The enzyme in dog serum was similar to that in pancreatin in that it required bile salts for activity, was inactivated by heating at 65° for 15 minutes, and had the same optimum pH for the hydrolyzing (pH 6.4) and esterifying (pH 6.1) systems. Other experiments confirmed earlier reports that the free cholesterol of human and dog sera was esterified during incubation and that the reaction was inhibited by bile salts. The nature and significance of this esterification reaction is discussed.

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THE NITROGENOUS CONSTITUENTS OF THE TISSUE LIPIDES

III. THE EFFECT OF ACUTE CHOLINE DEFICIENCY ON THE TISSUE LIPIDES OF YOUNG PUPPIES*

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Much information is available on the nutritional significance of choline and the physiological and pathological effects of choline deficiency. However, there has been much less study of the fundamental biochemical changes resulting from this deficiency. The extensive infiltration of neutral fat into the liver has suggested alterations in neutral fat metabolism and presumably in the metabolism of the essential lipides.

Considerable evidence is now at hand to show that this is indeed the case. Most observers have found a significant drop in the content of liver (1-6) and kidney phospholipides (1, 2) in the choline-deficient rat, although dietary factors and the method of expression of concentration markedly affect the magnitude of the change. Decreases in lipide choline appear to account in part for the drop in liver phospholipides of the choline-deficient rat (2-4, 6, 7), although increases in the non-choline-containing phospholipides may partially offset this effect and materially alter the pattern of these lipides (4). Severe choline deficiency in rats was actually found, in one laboratory, to increase the choline content of liver, kidney, and carcass (8). Choline deficiency in young puppies is characterized by decreases in blood serum cholesterol which are slightly greater in the ester fraction (9, 10).

A marked reduction is found in the phospholipides of liver (11) and blood (12) of depancreatized dogs maintained with insulin. The liver lipide choline is, however, decreased only to the same extent as the total liver phospholipide (13). Liver cholesterol ester is greatly increased (11), while the blood cholesterol ester is decreased (12) in this induced choline deficiency.

Methods for the purification of tissue lipide extracts and analysis for total nitrogen, phosphorus, choline, and sphingosine have been reported from this laboratory (14, 15). The purpose of this paper is to report the results obtained from the application of these methods to the tissue lipides

of choline-deficient puppies. The data permit a comparison of the above biochemical findings in rats and depancreatized dogs with those of acutely deficient puppies, and an extension of the observations to a number of other tissues and to the behavior of the sphingolipides.

<div align="center">EXPERIMENTAL</div>

Acute choline deficiency was produced in three litters of weanling puppies with the following ration fed *ad libitum:* sucrose 595 gm. per kilo, purified casein (General Biochemicals, Inc., vitamin test) 100 gm., alcohol-extracted peanut meal 150 gm., cottonseed oil 65 gm., cod liver oil 20 gm., salt mixture 40 gm.,[1] K_2HPO_4 5 gm., Wilson's liver fraction L 20 gm., 34 per cent mixed tocopherols (Distillation Products, Inc.) 5 gm., thiamine
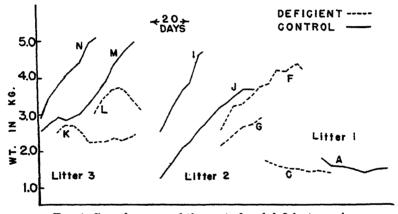
FIG. 1. Growth curves of the control and deficient puppies

hydrochloride 2 mg., pyridoxine hydrochloride 2 mg., riboflavin 4 mg., nicotinic acid 25 mg., folvite 5 mg., and calcium pantothenate 5 mg. This ration contains relatively normal amounts of protein (18.9 per cent) and fat (9 per cent) and is similar to previously reported Ration 5 (9). Five deficient and five control puppies (receiving 2 gm. of choline chloride per kilo of ration) were studied. Litter mate animals were placed on the rations simultaneously. The growth curves of these pups are shown in Fig. 1. The average over-all growth of the control puppies was 16.1 per cent of initial body weight per week, that of the deficient puppies 3.9 per cent. In order to include moderately deficient as well as severely deficient animals in the series, deficient Dogs G and F were sacrificed before severe deficiency occurred. The two pups in Litter 1 did very

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[1] The salt mixture described by Phillips and Hart (16) with an additional 1.20 gm. of $MnSO_4 \cdot 4H_2O$ per kilo of salt mixture.

poorly on the ration and may have been started on experiment prematurely. Since data from the tissues of these dogs appear to be comparable in most respects to those of the others, they have been included in the series.

All animals were sacrificed by intravenous injection of saturated magnesium sulfate solution and exsanguinated from the carotid artery under this deep anesthesia. In order to rule out changes in lipide composition of tissues due to aging (17), a litter mate control animal was sacrificed as soon after a deficient animal as was feasible. The tissue lipide extracts were prepared, purified, and analyzed for nitrogen, phosphorus, choline, and sphingosine as described previously (14, 15). However, nearly all of the hydrolyses were carried out for 7 hours instead of 5 hours with the saturated barium hydroxide solution, and some of the sphingosine extracts were analyzed for nitrogen by the micro-Kjeldahl procedure instead of with sulfuric acid and superoxol. All hydrolyses were performed in duplicate or until consistent maximal checks for choline were obtained. In addition, liver samples of all the dogs were dried and extracted with chloroform in Soxhlet extractors to determine the total liver lipide. This permits a rough assessment of the severity of choline deficiency (9). Values of total liver lipide for control Dogs A, I, J, M, and N were 7.1, 11.3, 11.4, 9.18, and 12.45 per cent of dry weight respectively. For the deficient Dogs C, F, G, K, and L, the values were 47.3, 35.5, 25.1, 47.6, and 38.7 per cent, respectively. Fatty infiltration was therefore moderate in Dog G and severe in all the other deficient animals. The livers of all the deficient animals appeared light to yellowish, greasy on section, and noticeably friable, whereas the control animal livers appeared grossly normal in all respects.

The analytical data are summarized in Tables I and II.

Results

No changes were observed in total lipide phosphorus or nitrogen in the livers of the deficient animals. However, a 29 per cent decrease in the choline nitrogen fraction of total lipide nitrogen occurred together with an increase in lipide sphingosine both on a gm. basis (38 per cent) and in the fraction of total lipide nitrogen (26 per cent). Total lipide phosphorus and nitrogen were reduced to about one-third of the control values in blood plasma. The per cent choline phospholipides of total phospholipides remained unchanged. The ratio of choline nitrogen to total lipide nitrogen was reduced (17 per cent), while that of sphingosine nitrogen was increased (31 per cent). In the intestine, total lipide phosphorus and nitrogen were decreased by 15 and 13 per cent respectively in the deficient animals. Lipide choline was decreased by 20 per cent on a gm. basis but the ratio of choline nitrogen to total lipide nitrogen was reduced only 9 per cent. Lipide choline in skeletal muscle of the deficient dogs was decreased 19

TABLE I

Blood Plasma, Intestine, Liver, and Skeletal Muscle Lipides in Choline Deficiency

Tissue		Lipide P	Lipide N	Lipide choline N	Per cent of total lipide N	Lipide sphingosine N	Per cent of total lipide N	Lecithin N	Per cent of total lipide N	Choline P Molar ratio	Sphingosine P Molar ratio	Extra N, per cent of total
Blood plasma	Control	323 ±36.5	391 ±44.9	296 ±32.2	76.1 ±1.01	45.7 ±4.27	11.9 ±0.47			0.919 ±0.0074	0.143 ±0.0057	5.52 ±0.70
	Deficient	102.2 ±26.3	169.1 ±37.5	93.1 ±24.4	62.9 ±5.24	23.0 ±4.28	15.6 ±1.11			0.898 ±0.016	0.253 ±0.031	14.77 ±5.32
	P	<0.01	<0.01	<0.01	0.04	<0.01	0.02			0.28	0.01	0.13
Intestine	Control	131.1 ±3.88	158.7 ±5.74	66.9 ±2.24	42.2 ±0.67	28.4 ±1.28	17.9 ±0.69			0.511 ±0.009		
	Deficient	111.6 ±2.51	138.8 ±6.01	53.5 ±2.73	38.5 ±0.378	28.6 ±1.15	20.7 ±1.16			0.479 ±0.020		
	P	<0.01	0.05	<0.01	<0.01	>0.90	0.08			0.19		
Liver	Control	159.6 ±17.1	170.2 ±18.3	85.9 ±9.92	50.4 ±1.71	17.1 ±2.39	9.9 ±0.53	68.8 ±7.85	40.5 ±1.77			
	Deficient	172.7 ±9.06	189.1 ±10.1	68.2 ±6.1	35.8 ±1.49	23.6 ±0.83	12.5 ±0.48	44.6 ±5.33	23.3 ±1.57			
	P	0.52	0.39	0.17	<0.01	0.04	<0.01	0.04	<0.01			
Skeletal muscle	Control	79.6 ±2.92	88.4 ±4.10	39.3 ±1.16	44.6 ±1.11	11.8 ±0.65	13.5 ±1.26					
	Deficient	70.3 ±3.67	75.6 ±4.66	32.0 ±1.92	42.6 ±1.74	12.3 ±0.75	16.4 ±1.35					
	P	0.09	0.08	0.01	0.36	0.63	0.16					

Concentrations for all the tissues except blood plasma are expressed in micromoles per gm. of dry, lipide-free tissue. Blood plasma values are expressed in micromoles per 100 ml. of plasma. Statistical calculations were made according to Fisher (22). The maximal P value considered significant is 0.05. The ± values are standard errors.

per cent on a gm. basis.   Little or no changes were observed in the lipides of lung, spleen, heart muscle, pancreas, kidney, and cerebrum.

TABLE II

Cerebrum, Heart, Kidney, Lung, Pancreas, and Spleen Lipides in Choline Deficiency

Tissue		Lipide P	Lipide N	Lipide choline N		Lipide sphingosine N	
					Per cent of total lipide N		Per cent of total lipide N
Cerebrum	Control	634	838	261	31.3	280	32.3
		±19.5	±38.6	±7.43	±0.91	±6.85	±1.37
	Deficient	633	815	256	31.5	260	31.7
		±14.7	±29.2	±8.08	±0.45	±14.7	±0.73
	P	>0.90	0.65	0.66	0.85	0.27	0.71
Heart	Control	159.5	156.9	72.9	46.7	23.0	14.8
		±5.23	±6.20	±0.71	±1.67	±0.88	±1.08
	Deficient	156.9	157.0	70.6	45.0	25.6	16.4
		±5.55	±7.20	±2.83	±0.52	±0.99	±1.24
	P	0.74	>0.90	0.45	0.36	0.09	0.36
Kidney	Control	198.3	222	90.9	40.9	41.7	18.7
		±4.35	±8.20	±3.08	±0.31	±2.05	±0.79
	Deficient	205	232	91.4	39.5	47.0	20.3
		±4.40	±6.28	±2.44	±0.52	±2.45	±1.02
	P	0.31	0.36	>0.90	0.05	0.14	0.25
Lung	Control	193.7	229	111.4	48.7	45.7	20.0
		±3.82	±4.94	±4.66	±1.08	±1.35	±0.34
	Deficient	182.1	223	99.6	44.9	46.7	21.2
		±10.7	±14.5	±4.99	±1.87	±3.10	±0.97
	P	0.34	0.72	0.13	0.12	0.78	0.29
Pancreas	Control	218	223	119.4	53.0	27.6	12.4
		±7.02	±10.2	±5.32	±0.53	±2.29	±0.60
	Deficient	211	211	119.7	56.8	26.2	12.4
		±3.10	±4.14	±2.64	±0.56	±2.13	±0.78
	P	0.40	0.33	>0.90	<0.01	0.67	
Spleen	Control	135.5	163.8	69.7	42.5	33.4	20.3
		±6.52	±6.65	±3.53	±1.33	±3.04	±1.72
	Deficient	129.3	164.0	64.4	39.3	32.8	20.0
		±4.78	±8.33	±4.13	±1.66	±0.82	±1.01
	P	0.47	>0.90	0.37	0.66	0.86	0.89

Concentrations for all tissues are expressed in micromoles per gm. of dry lipide-free tissue.   Statistical data as in Table I.

DISCUSSION

The observed maintenance of liver lipide phosphorus and nitrogen in our choline-deficient pups is partly due to the expression of these com-

ponents in terms of micromoles per gm. of dry lipide-free tissue. When
the infiltration of fat is very great, tissue substance, including water
(11, 2), becomes replaced by fat droplets. The expression of essential
lipide values in terms of dry lipide-free tissue tends to rule out this dilu-
tion effect of the neutral fat and permits a comparison of deficient and con-
trol animal tissues on the basis of cellular metabolic substance. Never-
theless, rat liver shows a reduction in total phosphatides in this deficiency
on a fat-free basis (2, 3, 5), although this effect is brought out more clearly
on a high fat diet (3). The shift in the pattern of the liver phosphatides
of the choline-deficient pups to a mixture of less choline and more non-
choline-containing phosphatides is similar to that observed by Patterson
and McHenry (2), Fishman and Artom (3, 4), and Stetten and Grail
(6) in the choline-deficient rat.

Liver lipide sphingosine has been considered as derived exclusively
from sphingomyelins, since others have shown no cerebroside in beef
liver (18) or dog liver (19), and since lignoceryl sphingosine (20, 21) has
not been demonstrated in dog liver. The absolute increase in sphingo-
myelins in the deficient livers is an unexpected response to this deficiency
since sphingomyelin is a choline-containing phosphatide. On the above
assumption we have calculated values for liver lecithin by subtracting
sphingosine values from total choline. The decrease in liver lecithins in
the deficient dogs thus becomes significant both in absolute quantities
per gm. of tissue (averaging 35 per cent) and in per cent of total phos-
pholipide (averaging 43 per cent). There is, however, considerable varia-
tion in the amounts of lecithin per gm. of tissue. Deficient Dogs F and
L had higher lecithin values on this basis than did control Dog A. Dog
L had almost as much as control Dog J. Both Dog F and Dog L had,
nevertheless, moderate to severe fatty infiltration of the liver. When
expressed as per cent of total lipide nitrogen, the lecithin nitrogen values
of the control and deficient dogs are confined to two sharply defined ranges.
A statistical t value (22) of only 2.42 is found for the decrease in liver
lecithin per gm., whereas this value is 7.27 for the decrease in lecithin
nitrogen as per cent of total nitrogen. Evidently the absolute amount of
lecithin present per gm. of liver by itself is of less significance than the
pattern in which all the phospholipides are laid down. Although our
series is small, it may be suggested that fatty infiltration of the liver may
occur when the total lipide choline nitrogen becomes lower than roughly 40
per cent of total lipide nitrogen of the liver, or when lecithin nitrogen falls
below roughly 30 per cent of total lipide nitrogen of the liver, irrespective
of the actual amount of lecithin per gm. of dry lipide-free tissue.

The mechanism by which neutral fat infiltration may be related to the
pattern of liver phosphatides is obscure. If the metabolic error is a failure

to metabolize neutral fat oxidatively, then the relationship between tissue phosphatides and the activity of oxidative enzymes becomes important. Liver cell mitochondria which contain the oxidative enzymes are rich in phosphatides (23). The turn-over rate of liver phosphatides is significantly increased following the administration of choline and fat (24) and decreased in choline deficiency (1, 5, 25). Recently Ball and Cooper (26) found a phospholipide content of 37 per cent in their most highly purified preparation of succinoxidase from beef heart. The necessity of a certain proper ratio of lecithins, cephalins, and sphingomyelins for full activity of these enzymes would not appear unreasonable.

There was no change in the percentage distribution between the choline and the non-choline-containing phosphatides of the blood plasma in this deficiency. Within the choline fraction, however, the deficient plasmas had a per cent increase in sphingolipides, presumably sphingomyelins, and a consequent decrease in lecithins. These changes reflect those occurring in the liver, which appears to be the principal source of the plasma phosphatides (27) and the principal organ concerned with their removal (28). A marked decrease in total plasma phosphatides was observed in depancreatized dogs maintained with insulin by Chaikoff and Kaplan (12), in choline-deficient rats by Handler (29), and in human patients with portal cirrhosis by Man et al. (30).

If the nitrogenous lipides of plasma consisted entirely of a mixture of monoaminophosphatides, sphingomyelins, and traces of cerebrosides, then the micromoles of total lipide nitrogen would always be equal to the sum of the micromoles of lipide phosphorus and sphingosine. However, the plasma lipide nitrogen values exceeded the sums of sphingosine and phosphorus by an average of 5.5 per cent in the control dogs and 14.8 per cent in the deficient dogs. Deficient Dog F had a lipide nitrogen "excess" of 29.2 per cent of total nitrogen. Whether or not this extra nitrogen is due to impurities not removed by our purification procedure remains to be established. Bile pigments tend to remain in the chloroform phase during purification with aqueous magnesium chloride, and these might contribute to this "extra" nitrogen, although the plasma of Dog F did not appear pigmented. In this case the excess lipide nitrogen would necessitate a plasma bilirubin level of 9.7 mg. per 100 ml. to be completely accounted for by this pigment. Such a high level of pigment does not appear probable and the authors are inclined to consider this nitrogen to be of lipide origin. An investigation of plasma lipide nitrogen of several species is now in progress and should give more information on this question.

The decrease in the choline content of the muscle mass may be a significant feature in the choline economy of this species. Under the assumption that muscle comprises 35 per cent and liver 4 per cent of body

weight in dogs of this age, muscle should contain almost 3 times as much total phosphatide and choline phosphatide as liver.

The deficient pup shows no kidney abnormalities (31), whereas extensive hemorrhagic degeneration occurs in young rats. Rat kidney phospholipides, on the other hand, are reduced nearly 50 per cent in terms of dry lipide-free tissue, and the total tissue choline is reduced by 26 per cent (2). This striking species difference in susceptibility to hemorrhagic degeneration of the kidney may well be due to the ability of the dog and the inability of the rat to conserve the kidney phosphatides.

The data as a whole indicate a notable maintenance of these tissue lipides in severe choline deficiency such as others have observed in severe caloric restriction (32–34). Marked changes occurred in only two of the tissues. The major change which does occur is the shift in the pattern of the liver lipides, which is associated with an impairment in the metabolism of neutral fat in the liver. The lipide pattern of each tissue in the control dogs was remarkably constant; more variation was observed in the absolute amount of such lipide laid down per gm. of tissue. Thus the standard deviation of the liver lipide choline per gm. of tissue was 25.8 per cent of the average value, and for lipide sphingosine 31.3 per cent. The standard deviation of choline nitrogen as per cent of total lipide nitrogen was only 7.76 per cent of the average value and that for sphingosine was only 12.0 per cent of the average. This constancy of pattern is noteworthy in all of the tissues studied, and it exceeds that of the absolute amount per gm. in all of the tissues except cerebrum, skeletal muscle, and heart. The pattern of these lipides may thus prove to be more important than the actual amount in studies of their metabolism.

The tissue sphingolipides were rigidly maintained in this deficiency, especially in such tissues as liver, kidney, lung, and intestine, where sphingomyelins are the predominant sphingolipide (18). However, real decreases in sphingomyelins could be masked by increases in cerebrosides, sphingosine amides, or other sphingolipides. Hence, conclusive proof of the maintenance of sphingomyelins, a significant feature of the choline economy of the organism, is not available from this study.

The data demonstrate the existence of high phosphorus-low nitrogen-containing lipide or lipides in pancreas, kidney, and heart. The sums of the micromoles of lipide phosphorus and sphingosine exceed those of the total lipide nitrogen in these tissues. For a simple mixture of monoaminophosphatides, cerebrosides, and sphingomyelins these would be equal. This excess indicates that these substances comprise a minimum of 10 to 15 per cent of the lipide phosphorus. Such substances as the brain "diphosphoinositide" described by Folch (35), the beef heart "cardiolipin" described by Pangborn (36), or other phosphatidic acids could account for

this analytical discrepancy. We have precipitated a high phosphorus, low nitrogen lipide fraction from dog heart extracts with barium salts after the procedure of Pangborn (37). It seems probable that dog heart also contains a cardiolipin.

The authors are indebted to Miss Carol Thomas for valuable technical assistance in this work.

SUMMARY

Total lipide nitrogen, phosphorus, choline, and sphingosine were determined on tissues of young choline-deficient puppies and their litter mate controls. In the deficient dogs a marked shift in the pattern of the liver lipides occurred, characterized by a decrease in choline and an increase in sphingosine and other undetermined nitrogenous components. It appears therefore that liver lecithins are decreased and the sphingomyelins increased, although no decreases in total phospholipides occur. Blood plasma total phospholipides were markedly decreased, but the percentage of choline phospholipides was maintained. A relative increase in the sphingosine nitrogen fraction suggests that sphingomyelins are replacing lecithins in the plasma. The lipide phosphorus and nitrogen of intestine were decreased and this decrease was only partly accounted for by a decrease in lipide choline. The lipide choline in skeletal muscle was decreased. Little or no change was observed in the lipides of the cerebrum, spleen, pancreas, kidney, heart, and lung.

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LIPOLYTIC ACTIVITY OF ADIPOSE TISSUE IN MAN AND RAT*

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In the course of a study of the factors involved in the production of lipodystrophies (fat atrophies and hypertrophies) by the subcutaneous injection of insulin (1, 2), it was considered pertinent to investigate the lipolytic activity of adipose tissue. Whereas data are numerous concerning lipases and esterases of other origin (3), references to the lipolytic enzymatic activity of adipose tissue are rather scanty. Quagliarello (4) in 1932 described an enzymatic splitting of both triolein and tributyrin by an extract obtained from fatty tissue of the dog. The activity of dried adipose tissue was found to be 1/200 that of the same weight of dried pancreas but 20 times that of the same weight of dried liver. Using a histochemical method, Gomori (5, 6) found evidence of lipolytic activity in the adipose tissue of the rat and rabbit, but none in that of other animals, and only very occasionally in man. The lipolytic activity of adipose tissue in man was noted and briefly discussed by Marble and Smith (2) and its presence indicated by Oesterreicher (7).

Methods

Extraction—In the albino rat, tissue was taken from the fat pads of the groin and the interscapular region. In the human subjects, the subcutaneous and articular adipose tissue was obtained from amputated legs by careful dissection from visible veins and fascia. Immediately after the death of the animal, or after amputation, the excised tissue was placed in a glass container surrounded with ice; all subsequent procedures were performed at or near 0°. The tissue was minced in a Waring blendor twice with 5 volumes of acetone and twice with 5 volumes of ether, then washed with ether on a filter, dried in partial vacuum in a cooled desiccator, and stored in a desiccator at 4°. The preparation of the dried tissue was completed within 2 hours of removal from the body.

In the process of extraction, the dry tissue was incubated for 1 hour at room temperature with 0.2 M borate buffer at pH 8.2 in the proportion of 500 mg. of tissue to 8 cc. of buffer for tissue of human origin, and

250 mg. to 8 cc. of buffer for that from rats. After centrifugation the slightly opalescent supernatant fluid was removed and used as an enzyme preparation. The protein content of the extract varied from 0.5 to 1.5 per cent and the non-protein nitrogen between 23 and 26 mg. per 100 cc. (fourteen extracts).

Determination of Lipolytic Activity—In the method of Archibald (8), used throughout the study, a mixture of polyoxyalkylene derivatives of an ester of 1 mole of fatty acid (90 per cent lauric acid) per mole of sorbitan (Tween 20)[1] is used as a substrate. This substrate is water-soluble. The free fatty acids formed are separated with ether and titrated. For routine determinations no activators or inhibitors of any kind were used.

TABLE I

Influence of pH of Extracting Buffer on Extraction of Enzyme Preparation*

pH of extracting buffer	M.eq. fatty acid formed	
	A† ·	B†
6.0	0.046	0.025
7.0	0.067	0.024
8.0	0.073	0.028
9.0	0.048	0.020
10.0	0.005	0.003

* Extraction of the dry, defatted tissue (female rat, group weighing 120 to 160 gm.).

†The activity is expressed in milliequivalents of fatty acid formed per hour at pH 8.2 and 37°. A, extract of 31 mg. of dry, defatted tissue; B, amount of extract containing 0.5 mg. of nitrogen.

For each determination a blank value was obtained by incubating separately the extract and the buffered substrate. For each new tissue the destruction of all lipolytic activity by heat was confirmed by placing the extract in a boiling water bath for 30 seconds.

The lipolytic activity is expressed in milliequivalents of fatty acids formed per hour at 37°. Two values are given for every tissue: The first relates the activity to a standard amount (31 mg.) of dry, defatted tissue. The second value relates the activity to the extract containing a standard amount of extracted nitrogen (0.5 mg.);[2] as the non-protein nitrogen was found to vary only between 23 and 26 mg. per 100 cc. in fourteen different extracts, this value can be considered to express the activity of a standard

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[1] Atlas Powder Company, Wilmington, Delaware.

[2] The standard amounts used were chosen arbitrarily as corresponding to the 0.5 cc. of extract used: 31 mg. of dry, defatted tissue yielded 0.5 cc. of extract, and the average nitrogen content of this amount of extract was 0.5 mg.

amount of extracted protein. This was thought to eliminate differences of extraction present even with standard conditions and evidenced by the variations in protein content. Total nitrogen and non-protein nitrogen values were obtained by micro-Kjeldahl procedures.

For all pH determinations a Beckman pH meter was used, and the pH indicated in the tables is always the pH at the beginning of the incubation, determined separately in each incubated sample. To evaluate the conditions of extraction, the activity of a number of extracts obtained at different temperatures and after times of extraction varying between 10 minutes and 22 hours was determined. It was evident that time and temperature had but little influence on the extraction. However, room temperature was slightly more favorable and there was indication that extraction time over 6 hours should be avoided. The pH during extraction was of great importance, with a definite optimum in the neighborhood of pH 8.0 (see Table I).

The activity of the extract of a given amount of dried, defatted tissue was found to equal or surpass the activity of the equivalent amount of dried tissue itself, added directly to the incubation mixture. (For this comparison the samples were continuously shaken during incubation.) The standard deviation for a single determination of lipolytic activity, obtained from twelve different extracts of the same tissue, was ±0.0018 m.eq.

Results

Lipolytic Activity of Adipose Tissue of Man and Rat—As illustrated by Fig. 1, *A* and *B*, the relation between the amount of enzyme present and the milliequivalents of acid formed can be considered linear up to a titration value of 0.04 m.eq. of fatty acid formed. At this point the pH of the sample is approximately 0.3 lower than at the beginning of the incubation, and is therefore getting outside the optimum pH range. If higher titration values are obtained, adequate dilution of the enzyme preparation with extracting buffer will bring the value into the desired range of linear relationship. The actual activity of the extract can then be calculated by taking into consideration the dilution used.

The pH optimum (Fig. 1, *D*) is in agreement with the findings of Quagliarello (4).

The lipolytic activity of the dry tissue, stored in a desiccator at 4°, has in no case shown a decrease exceeding 10 per cent over a period of 4 months. The lipolytic activity of the extract stored at 4° is stable up to a period averaging 3 weeks.

Relative degree of lipolytic activity of rat organs, as measured by Archibald's method, was obtained by comparing it with the lipolytic activity of similar

extracts of other organs. This was done separately for male and female rats. There was, however, no appreciable sex difference in the lipolytic activity of the tissues examined. The values obtained were within a range sufficiently narrow to justify their report as averages; these are represented graphically in Fig. 2.
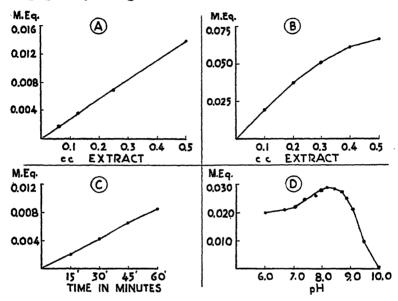
FIG. 1. Lipolytic activity of adipose tissue. In A and B is shown the relation of the amount of extract present to the amount of ether-soluble acid liberated from the substrate per hour at pH 8.2 and 37°. The amount of acid liberated is expressed in milliequivalents. A, extract of human adipose tissue (female patient J. P.). B, extract of rat adipose tissue (female, group weighing 220 to 250 gm.). The lipolytic activity of rat adipose tissue is significantly greater than that of human adipose tissue even if the nitrogen content of the extract is taken into consideration. C, the relation of the time of incubation with an extract of human adipose tissue to the amount of ether-soluble acid liberated from the substrate at pH 8.2 and 37°. D, the relation of the lipolytic activity of an extract of human adipose tissue to the pH of the reaction mixture at 37°.

Lipolytic Activity of Human Subcutaneous and Articular Adipose Tissue in Four Unselected Non-Diabetic and Ten Diabetic Patients—In the four non-diabetic patients subcutaneous fat could be obtained only in the course of various surgical procedures. In the diabetic patients subcutaneous and intra-articular subpatellar fat was taken from amputated legs.

The results for the four non-diabetic and the ten diabetic patients are·

given in Tables II and III and are shown separately for males and females. The striking sex difference is found on statistical analysis to be highly
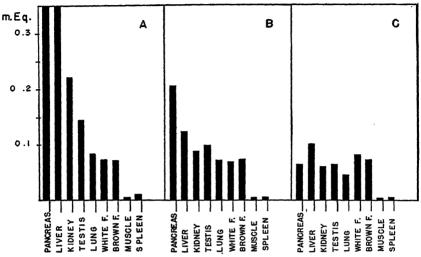
FIG. 2. Average lipolytic activity of various rat tissues. The tissues of five female animals weighing 220 to 250 gm. and of five male animals weighing 220 to 260 gm., pooled separately, were used for extraction. The activity is expressed in milliequivalents of fatty acids formed per hour at pH 8.2 and 37°. *A*, extract of 250 mg. of *fresh weight* of tissue; *B*, extract of 16 mg. of dry, *defatted* tissue; *C*, amount of extract containing 0.5 mg. of *nitrogen*.

TABLE II

*Lipolytic Activity of Subcutaneous Adipose Tissue of Unselected, Non-Diabetic Patients**

Patient	M.eq. fatty acid formed	
	A†	B†
Male (H. K.).................................	0.020	0.016
" (A. B.).................................	0.032	0.016
Female (A. H.).............................	0.044	0.043
" (G. M.).............................	0.048	0.040

* Extraction of the dry, defatted tissue with 0.2 M borate buffer at pH 8.2 and room temperature for 1 hour.
† Activity, see foot-note to Table I.

significant. Although it is not present in the rat (Table IV), it was thought of interest to compare the lipolytic activity of the brown (interscapular) and white fat of male and female rats at different ages and after a period

TABLE III

Lipolytic Activity of Adipose Tissue (Subcutaneous and Articular) of Five Male and Five Female Diabetic Patients*

Male patients	Type of adipose tissue	M.eq. fatty acid formed		Female patients	Type of adipose tissue	M.eq. fatty acid formed	
		A†	B†			A†	B†
B. S.	Subcutaneous	0.007	0.004	N. M.	Subcutaneous	0.021	0.013
	"	0.010	0.005	J. P.	"	0.018	0.019
	Articular	0.003	0.003		Articular	0.008	0.009
S. G.	Subcutaneous	0.004		L. A.	Subcutaneous	0.023	0.012
	"	0.009			"	0.017	0.011
	Articular	0.002			Articular	0.013	0.005
F. G.	Subcutaneous	0.010	0.006	M. B.	Subcutaneous	0.041	0.021
	Articular	0.004	0.003		"	0.053	0.024
R. S.	Subcutaneous	0.004	0.005		Articular	0.008	0.005
	Articular	0.002	0.001	E. B.	Subcutaneous	0.040	0.024
L. D.	Subcutaneous	0.006	0.006		"	0.042	0.027
	Articular	0.003	0.002		Articular	0.012	0.008
Average, subcutaneous......		0.007 ±0.003‡				0.029 ±0.005‡	
" articular.........		0.003 ±0.0003‡				0.007 ±0.001‡	

* Extraction of the dry, defatted tissue with 0.2 M borate buffer at pH 8.2 and room temperature for 1 hour.

† Activity, see foot-note to Table I.

‡ Standard error of the mean ($\sigma/\sqrt{n}$).

TABLE IV

Lipolytic Activity of Brown (Interscapular) and White (Subcutaneous) Fat of Rats at Different Ages and after a Period of Fasting*

Rats	No. of animals	Weight	Fatty tissue extracted	M.eq. fatty acid formed	
				A†	B†
		gm.			
Female.....................	20	70– 75	White	0.064	0.067
			Brown	0.036	0.033
"	7	120–160	White	0.075	0.038
			Brown	0.065	0.026
"	5	220–250	White	0.075	0.075
			Brown	0.066	0.066
Male......................	5	220–260	White	0.062	0.084
			Brown	0.080	0.075
Female fasted 72 hrs.........	4	130–170	White and brown	0.051	0.051

* Extraction of the dry, defatted tissue with 0.2 M borate buffer at pH 8.2 and room temperature for 1 hour.

† Activity, see foot-note to Table I.

of fasting. No correlation between lipolytic activity and sex, age, or food intake is apparent. In the young rat the brown interscapular fat (12) shows less lipolytic activity than the white fat.

DISCUSSION

From the data presented it appears justifiable to conclude that lipolytic activity of an enzymatic nature with a pH optimum at 8.2 is present in the adipose tissue of man and rat. The level of this activity is characteristic and surprisingly constant in any given tissue. This is in agreement with the uniformity of results obtained with the histochemical lipase determinations by Gomori (5), using an analogous water-soluble substrate, but is at variance with the greater scattering of values obtained with other methods in which the substrate must be emulsified and in which no separation of the fatty acids precedes their titration (9). In rats the lipolytic activity is approximately 6 times as great as in humans. In the rat the lipolytic activity of adipose tissue per unit of weight of dry, defatted tissue is equivalent to approximately one-third of that of the pancreas and one-half that of the liver. Of the organs examined, pancreas, liver, kidney, lung, and testis showed lipolytic activity; only muscle and spleen showed practically none. Of particular importance is the lack of lipolytic activity found in muscle and spleen, as this makes unlikely the possibility that the activity found in adipose tissue is derived from its content of blood, blood vessels, or some other generally distributed tissue.

These results apply, of course, only to the substrate used (Tween 20). However, since they agree with earlier results obtained with other substrates (9) and since they show a marked activity of both pancreas and liver, the substrate used seems to be well adapted to detect any kind of lipolytic activity.

In man the lipolytic activity of subcutaneous adipose tissue is approximately the same in normal subjects and in diabetic patients receiving amounts of insulin adequate for clinical control of diabetes. Our studies included, in the diabetics, the subpatellar, intra-articular fat; this adipose tissue belongs to a physiologically distinct group in which the storage function of fat seems to be subordinated to its mechanical function (plantar fat, the sucking pad of the new-born, retroorbital fat, etc.). In starvation this type of fat is characterized by its resistance to mobilization (10). It has further been demonstrated by early tagging methods with fat stains that the rate of turnover in this type of fat must be lower than in subcutaneous or abdominal fat (11). The lipolytic activity of the subpatellar, intra-articular fat was found in both males and females to be only one-third that of the subcutaneous fat.

The lipolytic activity in the male diabetic is consistently and surpris-

ingly about one-fourth that in the female diabetic, both in subcutaneous and in articular fat. In the four unselected, non-diabetic patients examined the difference was less striking, but still distinct. Other than sex, no factor which might account for the difference between the two groups was found. The difference noted may possibly be correlated with the clinical finding that the atrophic type of lipodystrophy due to insulin is found frequently in children and women but much less frequently in adult males (2).

Quagliarello (4) described an increased "autolytic fat splitting" in the fat of fasting dogs compared to fed dogs. In the rat we found no increase of lipolytic activity in the tissue of animals fasted for 72 hours compared with activity of the tissues of fed animals. In fact, lipolytic activity was somewhat lower in the fasted animals.

In conclusion, it should be emphasized that, while the lipolytic activity of a given group of adipose tissues is surprisingly constant, different groups of fatty tissue show quite clearly a number of distinct, reproducible differences which suggest that this lipolytic activity may have a distinct physiological function. The nature of this function is not apparent from data now available.

SUMMARY

1. Lipolytic activity of an enzymatic nature was demonstrated in the subcutaneous adipose tissue of both man and rat. In the rat such activity was approximately one-third that of the pancreas and one-half that of the liver.

2. In man the lipolytic activity of intra-articular fat amounted to only about one-third that of the subcutaneous fat.

3. In man the lipolytic activity of both subcutaneous and articular fat in males was consistently less than in females. In diabetic males it amounted to only about one-fourth of the corresponding activity in tissues of diabetic females.

4. In the rat there was no correlation between lipolytic activity and sex, age, or food intake. In the young rat the activity of the brown fat was less than that of the white fat.

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IMMUNOCHEMICAL STUDIES OF β-LACTOGLOBULIN*

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The ready availability and ease of crystallization of β-lactoglobulin have led to its use in many physical and analytical studies. The article of Li (1) on the presence of three electrophoretic components in β-lacto-globulin, when analyzed in buffers of pH 4.8 and 6.5, was the first definite report of the complex nature of this supposedly pure protein. Prior to this time variations in the physical properties of crystalline β-lactoglobulin, as described from various laboratories, had been suggestive of the inhomogeneity. The literature has been recently summarized by Lundgren and Ward (2).

Since quantitative immunochemical techniques are sensitive to protein inhomogeneity, it seemed advisable to study the properties of β-lactoglobulin by this technique. The great sensitivity of a serological reaction makes possible the detection of several antigens in a protein preparation. Because of the presence of these impurities, the quantitative course of the precipitin reaction between antigens, taken together, and the respective antibodies does not follow the usual course. However, if the main antigens are assumed to be classical β-lactoglobulin, a method of approximate assay for its content in milk whey is provided.

Materials and Methods

Electrophoretic analyses were carried out in buffers of 0.1 ionic strength. Experiments at pH 8.6 were performed in sodium diethyl barbiturate buffer. At all other pH values, NaCl constituted 80 per cent of the ionic strength, the remainder consisting of a univalent buffer ion. The position of moving boundaries was recorded with the standard Tiselius assembly by using the diagonal knife-edge schlieren method. β-Lactoglobulin samples used in these experiments were examined at 1.0 to 1.2 per cent concentration, whey samples over a range of 0.5 to 2.2 per cent.

β-Lactoglobulin—The preparations of Palmer (3) and of Cecil and Ogston (4) were used, the latter being essentially a combination of the procedures of Sörensen and Sörensen (5) and of Palmer. Because of its relative speed and simplicity, this method was used in the preparation of all but

three of the samples. These preparations were subjected to several re-crystallizations. Individual samples are designated by a number preceding an "X," followed by the letter (P) or (CO), to denote the methods of Palmer (P) and of Cecil and Ogston (CO). Thus, 8×(P) refers to a sample of β-lactoglobulin crystallized eight times, prepared by the method of Palmer. Solutions of this protein for immunochemical study were made up in borate buffer of pH 7.4 in saline, and merthiolate was added to a final concentration of 1:5000.

Whey—Fresh skim milk served as the source of the whey. The casein was removed by isoelectric precipitation. The pH of the whey was then adjusted to approximately 7.4, and the material was dialyzed overnight against running tap water and centrifuged to remove small amounts of insoluble material. The solutions were then either lyophilized or stored in the frozen state.

Rabbit Antisera—Rabbits were immunized by weekly subcutaneous injections of from 10 to 15 mg. of lyophilized protein suspended in paraffin oil until a suitable antibody response was obtained. Antisera to a 6×(P) and to a 5×(CO) preparation and to whey proteins were prepared. The antisera in each case were made up of the pooled serum of five animals and were brought to a merthiolate concentration of 1:5000.

Quantitative Precipitin Reactions—These reactions were carried out as previously described (6), except that the antigen-antibody mixtures were allowed to stand at 0-2° for 3 to 5 days prior to removal of the specific precipitates by centrifugation.

Assay of Supernatant Solutions—The solutions from the specific precipitates were studied qualitatively for both excess antigen and antibody. Approximately one-third of the solution was assayed for excess antigen and the remainder for excess antibody by the addition of small amounts of antiserum and antigen respectively. Following mixing, samples were allowed to stand at 0-2° for 24 to 36 hours. They were then centrifuged and observed for evidences of a specific precipitate.

Results

Electrophoretic Studies—In agreement with the report of Polis *et al.* (7), all of the crystalline β-lactoglobulin preparations studied in this investigation gave single electrophoretic peaks above the isoelectric point. At pH 4.2, these preparations showed from 20 to 30 per cent of a slower migrating component which was poorly resolved from the main peak. Polis *et al.* (7) indicate that their preparations contained approximately 40 per cent of this component of lower mobility. At pH 6.5, and after a previous dialysis for 36 to 48 hours at pH 4.2, a single electrophoretic component appears. The electrophoretic patterns at various pH values

for a 6×(P) sample are shown in Fig. 1. These patterns are typical of eight different crystalline β-lactoglobulins, prepared by the method of Palmer (3) or of Cecil and Ogston (4), each having undergone from two to eight crystallizations. Whey was likewise studied electrophoretically at a series of pH values to determine whether β-lactoglobulin in the original system showed evidences of more than one component, as well as to assay the level of this protein in the whey. The electrophoretic diagrams of
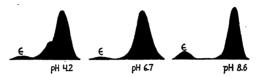
FIG. 1. Electrophoretic patterns of crystalline β-lactoglobulin at various pH values. Duration of experiments, pH 8.6, 182 minutes; pH 6.7, 240 minutes; pH 4.2, 180 minutes.
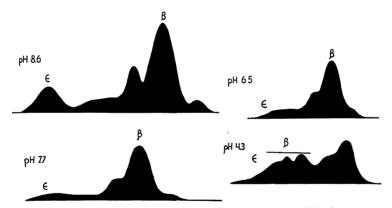
FIG. 2. Electrophoretic patterns of bovine whey at various pH values. Duration of experiments, pH 8.6, 180 minutes; pH 7.7, 245 minutes; pH 6.5, 209 minutes; pH 4.3, 203 minutes.

these preparations are shown in Fig. 2. They likewise reveal that β-lactoglobulin gives a single peak at pH values alkaline to its isoelectric point. At pH 4.3 several peaks are in evidence.

The analytical results for the amount of β-lactoglobulin calculated from the descending electrophoretic patterns of the whey sample (protein concentration 1.8 to 2.2 per cent) at pH 8.6, 7.7, and 6.5 (Fig. 2) give an average value of approximately 60 per cent. This may be compared to a value of 48 per cent previously found for whey at a concentration of 0.5 to 0.6 per cent when assayed in diethylbarbiturate citrate buffer at pH 8.6, ionic

strength 0.088 (8). The ratio of buffer salt to protein concentration in a mixture of proteins is a factor in determining the correct electrophoretic assay value for a given protein (9–13). Longsworth (13) has pointed out that the true composition of a protein mixture, as determined electrophoretically, may be obtained by extrapolation of the apparent composition either to zero protein concentration at constant ionic strength or to infinite salt concentration at constant protein content. The former method was used to determine the amount of β-lactoglobulin in whey when assayed in buffer of pH 8.6, ionic strength 0.1, over a relatively wide range of protein concentration. It should be noted that Smith (14) has found the apparent amount of β-lactoglobulin in whey, as determined in this manner, to be a function of the protein concentrations at constant ionic strength. The
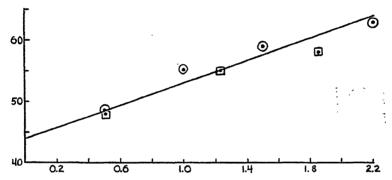
FIG. 3. Concentration of β-lactoglobulin in whey as a function of the protein concentration of whey. Data of Smith (14), ▫; present experiments, ⊙. Abscissa, per cent whey protein; ordinate, per cent β-lactoglobulin.

data obtained by us, as well as those reported by Smith (14), are plotted in Fig. 3. Extrapolation of the percentage of β-lactoglobulin in whey to zero protein concentration gives a value of approximately 44 per cent.

Immunochemical Studies, Rabbit Anti-6×(P)—Antiserum to this preparation of β-lactoglobulin was allowed to react with 4×(P), 8×(P), and whey. The experimental data are plotted in Fig. 4. Both of the preparations contain minor components, as indicated by the amount of specific precipitate formed upon the addition of large amounts of antigen (region of large excess of antigen). The 8×(P) sample showed a smaller amount of the major component as evidenced by the decreased specific precipitation upon the initial addition of antigen. The curve for the 8×(P) sample crosses that of the 4×(P) preparation upon larger antigen additions. This is indicative of the presence of larger amounts of these minor components in β-lactoglobulin 8×(P) than in 4×(P). The initial portion of the curve

does not represent the result of a reaction between a single antigen and its antibody. The supernatant solutions in this region show the presence of both antigen and antibody over a relatively wide zone.

The reaction of whey with this antiserum (Fig. 4) reveals that the various antigenic components in the two preparations of β-lactoglobulin studied exist in different ratios in the whey. A smaller amount of specific precipitate to whey protein results upon the addition of a large amount of these proteins. In the region from 5 to 300 γ of antigen N added, whey gives
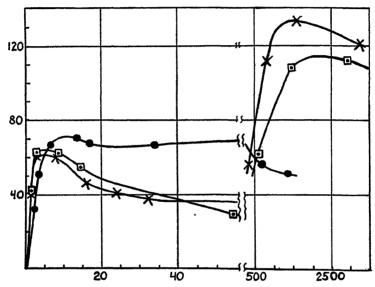
Fig. 4. Reaction of crystalline β-lactoglobulin preparations and whey with rabbit anti-β-lactoglobulin $(6\times(P))$ serum. $8\times(P)$, $\times$; $4\times(P)$, $\square$; whey, $\bullet$. Abscissa, micrograms of nitrogen in antigen added; ordinate, micrograms of total nitrogen precipitated.

considerably more specific precipitate than the β-lactoglobulin samples do. Upon further addition of protein, the whey curve gives evidence of being in the region of antigen excess, whereas the β-lactoglobulin samples are beginning to show maximum precipitation to the minor components. Such results are indicative of the presence in the whey of relatively larger amounts of the minor impurities as compared to the β-lactoglobulin preparations. It is interesting to note that the preparation crystallized eight times contains more of the minor components than does that crystallized four times. The results of the precipitin reactions of whey with this antiserum do not allow one to calculate the amount of β-lactoglobulin in whey.

Rabbit Antiwhey—This antiserum was employed since the above results point to the presence of small amounts of components (impurities) in the crystalline β-lactoglobulin preparations. Conceivably, the amounts of certain minor proteins might not be sufficient to elicit much antibody response. Their probable higher concentration in whey would elicit more antibody formation if the antigen employed was whey. Such an antiserum was allowed to react with six different crystalline β-lactoglobulins, including the 4×(P) and 8×(P) preparations employed previously. The two latter samples gave the same type of results for precipitin with this antiserum
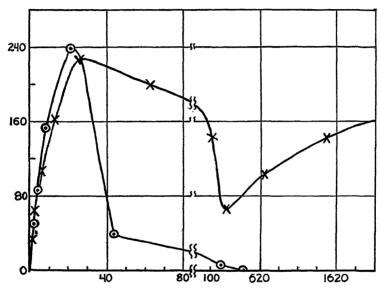
FIG. 5. Reactions of crystalline β-lactoglobulin preparations with rabbit anti-whey serum. 5×(CO), ⊙; 8×(P), ×. Abscissa, micrograms of nitrogen in antigen; ordinate, micrograms of total nitrogen precipitated.

as that shown in Fig. 4. The remaining β-lactoglobulin samples prepared by the method of Cecil and Ogston (4) gave little precipitation in the antigen excess region when they were allowed to react with rabbit antiserum to whey or to 5×(CO). In Fig. 5, the results of the reaction of 5×(CO) and 8×(P) with rabbit antiwhey serum are plotted. The curve for 5×(CO) was typical of four different crystalline β-lactoglobulin (CO) preparations. None of these gave an equivalence point. Excess antigen and antibody were detectable after the addition of as little as 1 γ of antigen N, whereupon approximately 20 γ of specific precipitate N resulted. The amount of specific precipitate, however, increased to over 205 γ of

N on the addition of 22 γ of antigen N, which is indicative of the reactions of several antigens with their antibodies.

Rabbit Anti-5×(CO)—The results of the reaction of 5×(CO) with its antiserum are shown in Table I and reveal that only a small amount of specific precipitation takes place in the region of extreme antigen excess. It should likewise be noted that no equivalence point is found. When the ratio of antibody N to antigen N in the region of antibody excess is plotted against antigen N, a straight line is not obtained. This type of finding is further indication of immunological heterogeneity (15) and of the presence of several proteins in crystalline β-lactoglobulin. An approximation of the ratio of antibody N to antigen N in the region of an equivalence point

TABLE I

Quantitative Precipitin Reaction of β-Lactoglobulin (5×(CO)) with Rabbit Antiserum to This Protein

β-Lactoglobulin N added	Ppt. N	Supernatant solution tests	
γ	γ		
0.65	17.4	Antibody excess	
1.65	38.8	"	"
3.3	69.0	"	"
6.6	117.3	"	antigen excess
15.9	236.7	Antigen excess	
31.9	106.7	"	"
63.8	46.1	"	"
311	18.0	"	"
622	7.9	"	"
3110	9.0	"	"
6220	13.5	"	"

gives a value in the neighborhood of 14. This is somewhat higher than might be expected for an antigen of molecular weight of 35,000 reacting with an antibody of molecular weight of 160,000.

The antiserum to 5×(CO) can be used to assay whey for its content of these proteins. The precipitin results for the reactions of 5×(CO) and whey with rabbit antiserum to 5×(CO) are shown in Fig. 6 and reveal that the curves for the reactions of these two proteins coincide in the antibody excess and slight antigen excess region if, in plotting the values for antigen added, the amount of whey nitrogen added is multiplied by 0.54. Thus, by quantitative immunological assay, whey contains 54 per cent β-lacto-globulin. This is higher than the value of 44 per cent obtained by extrapolation of the electrophoretic data (see Fig. 3). Although the β-lacto-globulin and the extrapolated whey curves coincide closely in the zone of

initial antigen addition, considerable variations in minor components are seen upon the addition of large amounts of antigens (Fig. 6).

Whey and two β-lactoglobulin preparations (4×(CO) and 8×(P)) were assayed against rabbit anti-β-lactoglobulin (5×(CO)) by the agar diffusion method of Oudin (16, 17).[1] Both of these preparations showed the presence of two components by this test. Whey gave evidence of four components when allowed to react with the same antiserum. This indicates that the 5×(CO) β-lactoglobulin preparation contains at least four antigenic components, two of which are present in much higher concentration in whey. The amounts of these minor antigens in the preparation
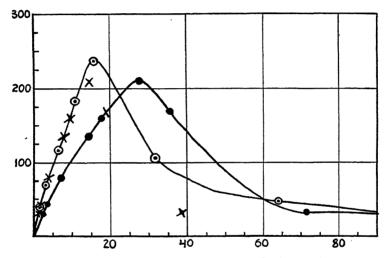
FIG. 6. Reactions of crystalline β-lactoglobulin and whey with rabbit anti-5× (CO) serum. 5 × (CO), ⊙; whey, ●; × values for whey nitrogen added × 0.54. Abscissa, micrograms of nitrogen in antigen added; ordinate, micrograms of total nitrogen precipitated.

(5×(CO)) are sufficient, however, to stimulate antibody production to them in the rabbit and thus to make possible their recognition when the antiserum is tested by the method of Oudin.

The rabbit antiserum to 5×(CO) gave a positive precipitin reaction with blood serum of a lactating cow. Quantitative assay revealed that a maximum of 26 γ of N could be precipitated per ml. of rabbit antibody in comparison with the maximum of 236 γ of N that was precipitated by 5×(CO), the homogeneous antigen. The results did not allow one to establish whether the bovine serum contained one of the components of the crystalline β-lactoglobulin as isolated from milk. It is interesting to note that

[1] The author wishes to thank Dr. Elmer Becker for carrying out these tests.

Palmer (3) likewise obtained immunological evidence for the presence of a serum component in crystalline β-lactoglobulin.

DISCUSSION

Quantitative immunochemical procedures reveal the heterogeneity and the marked variation of different samples of crystalline β-lactoglobulin. Preparations obtained by the method of Cecil and Ogston (4) appeared to be somewhat more uniform than those made by the method of Palmer (3). This difference was especially conspicuous in traces of minor components which gave a large amount of specific precipitate in the region of far antigen excess. The quantitative precipitin data do not allow one to determine the number of different proteins in the various samples studied. The method of Oudin (16, 17), however, indicates a minimum of four proteins.

The ability to assay a system such as whey for an antigenically heterogeneous protein by the quantitative precipitin method necessitates that the ratio of the major portion of the antigenic components in the protein used for immunization be the same as in the native state (18). The data of Fig. 5 reveal that the crystalline β-lactoglobulins prepared by the method of Cecil and Ogston (4) fulfil this requirement. Thus, the amounts of precipitin formed by the addition of various amounts of $5 \times$ (CO) and whey to rabbit anti-$5 \times$ (CO) serum gave identical results in the region of antibody excess if whey was considered to be 54 per cent β-lactoglobulin. This immunological value is considerably higher than the value of 44 per cent obtained by electrophoresis. A similar deviation is seen in the electrophoretic and immunological assay data for the amount of ovalbumin in egg white (6).

The observed immunological heterogeneity of crystalline β-lactoglobulin conforms with previous reports on its electrophoretic heterogeneity (1, 7, 19–22), and variations in solubility (23–24) and in the molecular weight as determined by several methods (25–28). In addition, some differences in the content of certain amino acids of different β-lactoglobulin samples are apparent (29, 30). Recent studies of this protein by paper chromatography by Franklin and Quastel have also revealed a number of components (31).

Polis *et al.* (7) have also reported the separation of a component of crystalline β-lactoglobulin which shows one electrophoretic peak at pH values acid, as well as alkaline, to its isoelectric point. Further studies of such preparations in the light of the available physical and biological techniques will be needed to reveal the relation of this protein to the parent crystalline entity.

SUMMARY

Crystalline β-lactoglobulin as prepared by several investigators shows pronounced immunological heterogeneity. Assay of whey by rabbit antiserum to this substance gives a value of 54 per cent. When whey is analyzed by electrophoresis, the component of mobility comparable to β-lactoglobulin constitutes 44 per cent of the protein in this system.

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DISSOCIATION CONSTANTS OF RADIUM-ORGANIC ACID COMPLEXES MEASURED BY ION EXCHANGE*

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In neutral media the cations of the alkaline earth elements form partially dissociated complex ions with many weak organic acids, particularly those involved in the tricarboxylic acid cycle. Quantitative measurements of the extent of this association are fundamental for the elucidation of the chemical, biochemical, and metabolic behavior of the alkaline earth and other elements. The dissociation constants of Mg, Ca, Sr, and Ba complexes with citrate or numerous other organic acids have been determined by methods such as the frog heart preparation (1), solubility, potentiometric titrations, and other electromotive force procedures. As far as we can ascertain, no measurements of the dissociation constants of radium complexes have been reported. This is understandable in view of the hazardous and destructive action of the radiations emitted by high radium concentrations. These difficulties are avoided in the ion exchange method (2) for measuring dissociation constants because less than microcurie quantities of one of the components is required. The ion exchange technique has been used for studying the citrate and tartrate complexes of alkaline earth and rare earth elements (3–5).

In this paper we shall report on the stability of the complexes formed between radium and several organic acids. In addition, we shall present an improved formulation of the equations used for calculating dissociation constants from ion exchange equilibria.

Pertinent Equations

The dissociation reaction for the complex ion, $(M_xA_y)^c$, or its equivalent, $(MA_{y/x})^c$, is

$$(1) \qquad (MA_n)^c \rightleftharpoons M^a + nA^b$$

In these expressions M and A represent cationic and anionic groups, x and y the number of such groups in the complex ion, a and b the charges on the

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dissociated cations and anions, respectively, c the net charge on the complex, and $n = y/x$. The dissociation constant, K_c, for the complex ion follows from the law of mass action:

$$K_c = \frac{(M^a)(A^b)^n}{(MA_n)^c}$$

(2)

The equilibrium distribution of a tracer ion between the exchanger and the solution is defined by the expression

$$K_d = \frac{\% \text{ in exchanger}}{\% \text{ in solution}} \times \frac{\text{volume of solution}}{\text{mass of exchanger}} = \frac{M_s}{M_1} \times \frac{v}{m}$$

(3)

where K_d is the distribution coefficient. Let K_d^o and K_d represent the value of the distribution coefficient obtained in the absence and presence respectively of the specific complex.

Two measurements are necessary, one of K_d^o and one of K_d under identical conditions except for the presence of the agent forming the given complex ion. The complex ion, if of zero or negative charge, is not adsorbed. MR (R is the immobile anionic part of the cationic exchanger) must be in equilibrium with M^a even in the presence of the complex; hence $M^a = MR/K_d^o$, where M^a and MR represent concentration units of the cation in the liquid and exchanger phases respectively. This value of M^a is substituted into Equation 2. The value of $(MA_n)^c$ is equal to the total radioactivity remaining in solution at equilibrium less the activity provided by M^a. This is formulated as follows:

(4) $$M^a + (MA_n)^c = MR/K_d$$

(5) $$(MA_n)^c = MR/K_d - M^a = MR/K_d - MR/K_d^o$$

By substitution into Equation 2 and simplification, the following expression is given:[1]

$$K_c = \frac{(A^b)^n}{(K_d^o/K_d) - 1}$$

(6)

Equation 6 can be written in the linear logarithmic form,

(7) $$\log R = n \log A^b - \log K_c$$

where R represents the denominator of Equation 6.

A plot of R versus A^b on log-log paper should give a straight line with slope n. The numerical value of K_c may be obtained directly from the same plot since it is equal to $(A^b)^n$ divided by the corresponding ordinate value of R.

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[1] Equation 6 may be directly obtained in terms of the derivation given previously (2) by redefining the terms a and s so that $a = M_s/m$, and $s = M_1/v$. Since $K_d = a/s$ (2), Equation 8 becomes identical to Equation 6 given above.

It is possible to calculate K_d^o from K_d measured at any two different concentrations of the agent (all other conditions such as ionic strength and pH remaining constant). Thus, when the concentrations of A^b are A_1^b and A_2^b respectively, the value of K_d^o may be obtained by use of Equation 8.

$$(8) \qquad K_d^o = \frac{(K_d)_1(K_d)_2[(A_1^b)^n - (A_2^b)^n]}{(K_d)_1(A_1^b)^n - (K_d)_2(A_2^b)^n}$$

The values of K_d must be independent of changes in either v/m or the concentration of tracer. This is generally the case since the tracer can be kept at concentrations of the order of 10^{-8} M or less. K_d, depending on the amount of ion exchanger employed, remains constant up to concentrations several magnitudes greater. The point at which inactive carrier or ionic impurities will cause an appreciable change in K_d is easily calculated (6).

A more serious consideration involving the constancy of K_d^o may occur in cases involving hydrolyzable ions, which in turn may lead to the *formation of colloids*. The presence of ions which may form insoluble compounds with one of the components of the complex ions may also lead to *colloidal formation*. In these cases the distribution coefficient varies with changes in v/m (2, 7). When the colloid is converted to a soluble complex, the distribution coefficient becomes independent of variations in v/m. All things considered, it is preferable to determine K_d^o by suitable extrapolation of a large number of K_d values. According to Equation 6, a linear relationship exists between $(A^b)^n$ and $1/K_d$. Extrapolation of the straight line to the point where $(A^b)^n = 0$ gives directly the reciprocal value of K_d^o. The proper value of n is that value which yields a straight line. The dissociation constant, K_c, is then calculated by substitution of the proper quantities into Equation 6. Therefore, one can by-pass the colloidal problem by conducting the experiment under such conditions that K_d^o and of course K_d are measured in a buffered solution containing a suitable agent which forms an auxiliary complex ion.

Agreement between extrapolated values of K_d^o and those determined by direct experiment is, in any event, a good check for internal consistency.

EXPERIMENTAL

Materials—The cation exchanger employed was Dowex 50 (8), which is an aromatic hydrocarbon polymer containing nuclear sulfonic acid groups as the sole active group at any pH value. The resin was classified into particles of 100 to 150 mesh. A large batch was treated alternately with approximately 10 per cent solutions of HCl and NaCl, then subsequently for 1 hour periods with fresh batches of saturated NaCl solutions. The sodium resin was placed in a column and 0.16 M NaCl was percolated

slowly through the bed until the pH of the effluent equaled the pH of the influent solution. Finally, the resin was washed free of adhering salts with distilled water and dried at room temperature. The air-dried resin had a moisture content of 18.25 per cent and contained 9.59 per cent of Na (4.17 m.eq. per gm., air-dried, or 5.10 m.eq. per gm., oven-dried).

The radium tracer used was Ra^{228} (MsTh₁). A 0.1 M HNO_3 solution of Ra^{228} was freed from daughter activities by coprecipitation with 2 mg. of Ba as $BaCl_2$ from an ether-HCl mixture (9). The $BaCl_2$ precipitate was dissolved in 100 ml. of 0.16 M NaCl adjusted to a pH of 7.2 with dilute NaOH solution. The radiochemical properties of the purified tracer agreed with those accepted for Ra^{228}.

All the solutions were prepared with chemically pure reagents.

Procedure—To 50.0 mg. of the sodium form of Dowex 50 were added 100 ml. of a solution containing carrier-free Ra^{228}, 0.16 M Na^+, about 0.16 M Cl^-, and a known concentration of an organic acid, all at pH 7.2 to 7.4. The pH was measured with a glass electrode. Each solution contained 1.00 ml. of the stock solution of tracer or about 20 γ (0.00003 m.eq. of Ba^{++}), and even smaller amounts of other cations, an insignificant amount relative to the capacity of exchanger employed ((6) pp. 176–178) or to the concentration of the organic anions.

The mixtures were contained in glass-stoppered Erlenmeyer flasks and agitated for 5 hours, except in the case of the citrate systems at 7° and 25°. In the latter the contact times were 50 and 2 hours respectively. Actually 2 hours contact gave a sufficiently reproducible constant adsorption, though a slight increase occurs between the 2nd and the 5th hour, as evidenced by the different K_d^o values obtained. However, since relative values are measured, small differences in the absolute values of K_d^o and K_d are inconsequential.

After the shaking period, the resin was allowed to settle and the bulk of the supernatant solutions was removed by aspiration. The resin plus a small amount of solution was transferred to a centrifuge tube with distilled water, centrifuged, and nearly all of the supernatant solution was removed. The resin was washed with 5 ml. of distilled water, transferred to a 10 ml. porcelain dish, and taken to near dryness by over-head heating from an infra-red lamp. A drop of mineral oil was added to prevent the resin particles from scattering, and the heating was continued until the resin mixture was free of moisture. The samples were counted through a 13.5 aluminum absorber by a thin end window Geiger tube. The stock solution of tracer was counted under identical conditions. The radiations counted were principally the 1.55 Mev β^- arising from the 6.13 hour Ac^{228} (MsTh₂) daughter of Ra^{228}. At least 4 days were allowed to elapse before the samples were counted so that radioactive equilibrium could be attained between the parent-daughter pair.

A number of factors were studied in order to test the reliability of the above procedures. First, it was shown that no adsorption of Ra[228] on the walls of the containing vessels took place. Secondly, the constancy of K_d to several fold changes in the v/m ratio was demonstrated. Thirdly, adsorption of Ra[228] by the exchanger was ascertained to be independent of the pH in the range 3.1 to 8.8. Finally, material balances, determined by counting the activity in both the solution and resin phases, were found to yield 100 ± 3 per cent recoveries.
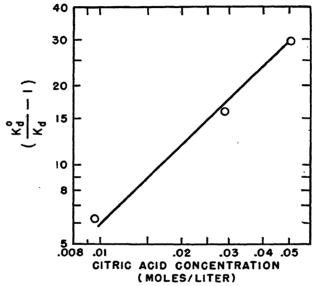
Fig. 1. Formation of the strontium citrate complex ion. Variation of $(K_d^o/K_d) -1$ with citric acid concentration ($t = 25°$, pH = 7.2 to 7.4, $\mu = 0.16$).

Results

Strontium Citrate—As a check on the absolute accuracy of the measurements, a determination of the well known dissociation constant of strontium citrate was made. Previous studies showed that Sr^{++} reacts with the tertiary citrate anion, Cit[≡], to form the complex ion (SrCit)[-]. The dissociation constant follows from the law of mass action.

$$(9) \qquad K_c = \frac{(Sr^{++})(Cit^{\equiv})}{(SrCit)^-}$$

At an ionic strength of 0.15 to 0.16 and neutral pH, the pK_c was found to be 2.92 by an amalgam electrode method (10), 2.70 by the frog heart method (1), and 2.81 by the ion exchange method with the cation exchange resin, Amberlite IR-1 (3).

In the present experiment, a value of K_c of $1.7 \pm 0.1 \times 10^{-3}$ or a pK_c of 2.8 for the (SrCit)$^-$ complex was found (Fig. 1). The experimentally

TABLE I

Ion Exchange Data on Interaction of Radium with Organic Acids

Solution, 100 ml., pH 7.2 to 7.4, about 0.005 μc. of Ra^{228}, total cation concentration furnished by Na$^+$ and adjusted to Na$^+ = 0.16$ M by addition of NaCl, organic acid concentration as tabulated. Resin, Dowex 50, 50.0 mg., sodium form, 100 to 150 mesh.

t	Organic acid	Molar concentration of acid	Ra^{++} adsorption	K_d	K_d^0 (extrapolated)	K_c ($\times 10^3$)	Average K_c ($\times 10^3$)	pK_c
°C.			*per cent**					
7	Citric	0.0050	68.3 ± 1.6	4.31	7.05	7.87		
7	"	0.010	63.8 ± 2.0	3.52		10.0		
7	"	0.020	56.4 ± 1.2	2.58		11.5		
7	"	0.030	47.5 ± 1.3	1.81		10.4	9.9 ± 1.0	2.0
25	"	0.00	74.6 ± 0.4	5.84	6.10			
25	"	0.0020	71.1 ± 0.7	4.87		8.16		
25	"	0.0050	66.9 ± 1.0	4.00		9.67		
25	"	0.0080	60.2 ± 0.4	3.02		7.90		
25	"	0.010	59.2 ± 0.8	2.90		9.00		
25	"	0.015	55.6 ± 0.3	2.50		10.4		
25	"	0.020	46.3 ± 0.7	1.76		7.85		
25	"	0.030	43.0 ± 0.8	1.50		9.78	9.0 ± 0.9	2.0
37	"	0.00	79.2 ± 1.2	7.62	7.24			
37	"	0.0020	75.1	6.04		10.0		
37	"	0.0050	71.0	4.90		10.4		
37	"	0.010	67.8	4.20		13.9		
37	"	0.020	56.1	2.51		10.6		
37	"	0.030	50.0	2.00		11.5	11 ± 1	1.9
25	" †	0.00	70.0 ± 0.05	4.66				
25	"	0.015	62.7 ± 0.05	3.36		3.75‡		2.4
25	Tartaric	0.0020	76.5 ± 1.0	6.50	6.71	63		
25	"	0.010	74.2 ± 0.4	5.74		59		
25	"	0.020	71.8 ± 0.3	5.08		62		
25	"	0.030	66.1 ± 0.05	3.90		42	57 ± 7	1.2
25	Succinic	0.0020	77.9 ± 0.4	7.03	6.95			
25	"	0.0050	76.6 ± 2.1	6.55		81		
25	"	0.010	75.9 ± 0.3	6.29		95		
25	"	0.020	74.7	5.90		112		
25	"	0.030	73.5	5.55		120	100 ± 14	1.0

TABLE I—*Concluded*

t	Organic acid	Molar concentration of acid	Ra⁺⁺ adsorption	Kd	K_d^0 (extrapolated)	K_c (× 10³)	Average K_c (× 10³)	pK_c
°C.			*per cent**					
25	Aspartic	0.0020	76.1 ± 0	6.36	6.60	54		
25	"	0.0050	76.1 ± 0	6.36		135		
25	"	0.010	75.9 ± 1.4	6.30		208		
25	"	0.020	74.0 ± 1.0	5.71		131		
25	"	0.030	73.8 ± 0	5.63		175	140 ± 40	0.86
25	Pyruvic	0.020	74.3 ± 1.3	5.80	6.75§	130‡		0.89
25	Oxalacetic	0.0064	70.3 ± 0.6	4.74	6.75§	15‡		1.8
25	Fumaric	0.020	65.2 ± 1.3	3.75	6.75§	25‡		1.6
25	Sulfosalicylic	0.010	65.0 ± 1.0	3.72	6.75§	12‡		1.9

* Each point represents the average of at least two separate runs, except when no standard error is reported. In the latter cases only one run was made.

† The solvent contained 38 per cent by weight of ethyl alcohol.

‡ Dissociation constant, calculated on the assumption that at a 1:1 ratio the complex was formed.

§ The K_d^0 reported for the acids from pyruvic to sulfosalicylic was determined directly in parallel experiments. The observed per cent adsorption was 77.3 ± 1.5.

determined value of 2.55 for K_d^0 agreed within experimental error with the value obtained by extrapolation of $1/K_d$ values to zero concentration of citric acid.

Radium Complexes—The data relating to the interaction of Ra⁺⁺ with the sodium salts of citric, tartaric, succinic, aspartic, pyruvic, oxalacetic, fumaric, and sulfosalicylic acids are given in Fig. 2 and Table I. The complexes appear to be of the 1:1 type, *i.e.* (Ra A)²⁻ᵇ.

At 25° the stability of the complexes[2] is in the order (Fig. 2), citric > sulfosalicylic > oxalacetic > fumaric > tartaric > succinic > pyruvic > aspartic.

No appreciable variation of the dissociation constant of radium citrate (RaCit)⁻ with temperature was found in the range 7–37°. Between 15–38° Hastings *et al.* (1) found no important change in the K_c of calcium citrate. These results are reasonable in view of the small effect of temperature on the dissociation constants of citric acid and the citrate anions, as shown in the careful work of Bates and Pinching (12). For example,

[2] Oxalacetic and other β-keto acids undergo spontaneous decarboxylation in aqueous solution (11). For this reason, the K_c observed for the radium-oxalacetic acid complex may be too high. In any event, measurements of the K_c of β-keto acids should be made at a sufficiently low temperature so that the rate of decarboxylation is negligible relative to the time necessary for the ion exchange equilibrium to be completed.

from 0–50° the dissociation constant, pK_2, of the primary citrate ions, $(H_2Cit)^-$, decreased from 4.837 to 4.757 (12).

The effect of a change in dielectric constant of the solvent on the K_c of radium citrate is shown by the results of measurements made in 38 per cent by weight of ethyl alcohol (Table I). In the latter solvent the dielectric constant relative to water is 0.7. As expected (13), the complex ion has less tendency to dissociate in ethanol than in water.
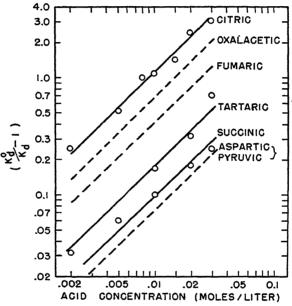
Fig. 2. Formation of radium complex ions. Variation of $(K_d^o/K_d) - 1$ with acid concentration ($t = 25°$, pH = 7.2 to 7.4, $\mu = 0.16$).

DISCUSSION

Effect of Citric Acid on Urinary Radium Excretion—The citric acid complexes of cations are easily diffusible and hence are available for excretion through the membranes of the kidney and bowel, as was pointed out by Kety in his studies on lead citrate (14). From the metabolic point of view it is of interest to calculate the extent to which the normal citrate content of human plasma can form a complex ion with circulating radium. In making this calculation we shall employ Kety's (14) procedure. The total citrate content of plasma is taken as 0.00012 M, that of Ca^{++} as 0.0013 M. The available citrate is corrected for that amount bound by Ca^{++}. For

the latter calculation a value of 6.03×10^{-4} is taken for the K_c of $(CaCit)^-$ (1).

Calculation shows that normally only 0.4 per cent of circulating radium could be bound by citrate and hence available for excretion. In chronic radium poisoning in humans the blood content averages about 0.03 per cent of the total amount fixed in the body (15). The kidneys filter about 170 liters of blood in 24 hours. Taking the average total blood volume to be 5 liters, we would expect that the amount of radium excreted via the kidneys would be $(170 \times 0.03 \times 0.004)/5 = 0.004$ per cent per day. This figure is about 10 times greater than that found (15); hence it could be concluded that about 90 per cent of the radium passing through the kidney membranes is reabsorbed. The calculation assumes that a relatively small
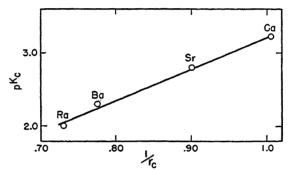
Fig. 3. Relationship between the dissociation constants $(-\log K_c)$ of the citrate complexes $(t = 25°, \mu = 0.16$ to $0.2)$ of the alkaline earths and the cationic radii $(1/r_c)$.

amount of Ra^{++} is incorporated into complex ions by the other blood constituents.

The effect of citrate administration as a means of increasing radium excretion can be estimated. An intravenous injection of 50 ml. of a 3 per cent sodium citrate solution could raise the blood citrate level to 0.001 M, at which point about 2 per cent of the circulating radium (35 per cent of the Ca^{++}) would be bound. Even if such a blood level could be maintained continuously for 24 hours, the urinary excretion of Ra could increase only 5-fold. However, the excess citrate disappears from the blood stream well within 2 hours after intravenous administration; therefore it can be concluded that sodium citrate administration is without value for reducing the concentration of Ra in the body.

Factors Involved in Complex Ion Formation—By analogy with the Born expression, which relates the free energy of hydration of cations to their cationic radii (16), a plot of pK_c *versus* the inverse of the cationic unhy-

drated (crystal) radii was made for the citrates of Ca^{++}, Sr^{++}, Ba^{++}, and Ra^{++} (Fig. 3).[3] The radii (A) used in Fig. 3 were as follows:[4] Ca^{++} — 0.95, Sr^{++} — 1.11, Ba^{++} — 1.29, Ra^{++} — 1.37. The decreasing order of stability of the alkaline earth-organic acid complex ions with increasing crystal radius beginning with Ca^{++} has been generally found to hold in a large number of cases (17, 18). The stability of the citric acid complexes of the rare earths also decreases as the crystal cationic radii increases (6).

Inasmuch as the above systems are in an aqueous media, the cations are hydrated. Hence, it is the hydrated cationic radii that describe the "size" of the cation. The hydrated radius can be evaluated in a relative sense by means of the Debye-Hückel parameter $\hat{a}$, which is defined as the mean distance of approach of the ions, positive or negative (19). With $\hat{a}$ as the index of ionic hydration, it follows that those cations with the smallest crystal radius have the largest hydrated radius. On this basis the stability of the complex citrates of the alkaline earths would increase as the crystal radii increase. The apparent anomalous behavior observed with the complex ions is found with anions that are proton acceptors. A reasonable explanation is suggested by Harned and Owen ((19) p. 385) to account for similar reversals in "normal order" found for the activity coefficients of hydroxides, acetates, etc. They suggest that the protons in the hydration sheath surrounding the cation are repelled and tend to form linkages with proton acceptors such as hydroxyl and carboxyl ions. This mechanism leads to the conclusion that the binding between a cation and proton acceptor will be greater, the smaller the crystal radius of the cation; i.e., the protons in the hydration sheath are repelled to a greater extent and hence hydrogen bonding is facilitated. When the radius becomes quite small, the separation of the proton from the hydration sheath becomes so great (i.e., increased hydrolysis) that hydrogen bonding becomes relatively weak. In such cases, as with Mg^{++}, irregularities in the order of dissociation constants occur (17). Until more data are available it is preferable to withhold more detailed speculations and calculations, especially those pertaining to the transition elements, since factors relating to electronic structure, disregarded in the above discussion, become increasingly important. It might be noted that the organic acid complex ions formed by elements such as Mn^{++}, Ca^{++}, Ni^{++}, and Cu^{++} are considerably more stable than those formed by the alkaline earth elements; e.g., the malonates (20).

The presence and relative location of certain groups or groupings in the anion are important factors in determining stability. Of interest is the

[3] The dissociation constants for these complexes were obtained from the following sources: Ca^{++} (1), Sr^{++} (3), Ba^{++} (4), and Ra^{++} from the present investigation.

[4] W. H. Zachariasen, private communication.

work of Muus and Lebel (21), who measured the dissociation constants of calcium citrate and calcium tricarballylate (tricarballylic acid differs from citric acid in having a hydrogen atom in the place of the hydroxyl group). They found that the citrate complex was 50 times more stable than the tricarballylate, which emphasizes the importance of the hydroxyl group. The extent of enolization facilitates complex formation, as shown by the relatively weak complex formed between radium and pyruvic acid on the one hand and the strong complex formed between radium and the readily enolizable oxalacetic acid on the other.

We are now engaged in the determination of the dissociation constants of organic acids, particularly those involved in the tricarboxylic acid cycle, with cations such as Ca^{++}, Cu^{++}, Mn^{++}, and Co^{++}. It is expected that the data will clarify many of the factors relating to complex ion formation and elucidate the mechanism of action of trace metals in biochemical reactions; for example, in the metal-catalyzed decarboxylation of β-keto acids (11).

SUMMARY

1. A modified formulation of the equations used to calculate the dissociation constants by the equilibrium ion exchange method has been presented.

2. The dissociation constants of the complexes formed between radium and several acids (in isotonic neutral media) were measured. The complexes are of the 1:1 type. At 25° the stability of the radium complex ions is in the order citric > sulfosalicylic > oxalacetic > fumaric > tartaric > succinic > pyruvic > aspartic.

3. Between 7–37° no appreciable change in the dissociation of radium citrate is found.

4. A preliminary discussion is given of factors involved in the formation of complex ions. It is postulated that the protons in the hydration sheath surrounding the cation tend to form linkages with those anions that are hydrogen acceptors. Some relations of complex formation to metabolic and biochemical phenomena are also considered.

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SOME BIOLOGICAL AND CHEMICAL PROPERTIES OF THE CITROVORUM FACTOR

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Previous reports have indicated that certain natural materials will promote the growth of *Leuconostoc citrovorum* 8081 on a purified culture medium. Two substances, thymidine and an unidentified *citrovorum* factor (CF), were shown to contribute towards these growth-promoting effects (1–6).

Recent studies have indicated (5–8) that either CF or thymidine will reverse the growth inhibition of folic acid antagonists for some microorganisms under conditions in which folic acid (FA) is ineffective.[1] In other experiments high levels of FA were found to replace CF partially (1, 4). It has also been observed (9)[2] that rats and human beings given high levels of folic acid excrete levels of CF in the urine proportional to the folic acid dosage. All of these observations suggest that CF is a biologically active derivative of FA.

In the present study CF and thymidine were found to show competitive and non-competitive antagonism respectively in reversing the effect of 4-aminopteroylglutamic acid (4-amino-PGA) for inhibiting the growth of *L. citrovorum*. CF concentrates were found to be effective in reversing the toxicity of 4-amino-PGA for mice. As has previously been reported (10) FA was ineffective in reversing this toxicity.

Chemical and microbiological studies in this investigation showed that CF is structurally related to FA. A preliminary report of this work has appeared (11).

EXPERIMENTAL

Preparation of Citrovorum Factor (CF Concentrate)—Unless otherwise indicated, the CF concentrate used was prepared as follows: 30 kilos of a liver fraction derived from an alcohol-insoluble residue of a water extract of liver were suspended in 200 liters of water, 1.5 kilos of Magnesol (an adsorbent magnesium silicate) were added, and the mixture was stirred

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[1] In accordance with editorial policy, the term "folic acid" (FA) is used throughout this manuscript to designate pteroylglutamic acid.

[2] Welch, A. D., and Anker, R. M., private communication.

for 15 minutes at pH 3.5. The suspension was filtered and to the clear filtrate 0.5 per cent charcoal was added, followed by stirring for 30 minutes at pH 4.0. After filtration the charcoal was washed with 0.2 N sodium hydroxide and was then eluted with a mixture of 5 volumes of ethanol, 2 volumes of 14 N ammonium hydroxide, and 3 volumes of water. The eluate was concentrated to a small volume under reduced pressure and was stable for long periods of time when stored at 0° at pH 8.0. Typical CF concentrates obtained by this procedure contained 100,000 to 500,000 CF units (1) per ml. and 10 to 50 γ of folic acid activity for *Streptococcus faecalis* expressed as micrograms of FA equivalence.

Microbiological Methods—*L. citrovorum* 8081 and *S. faecalis* R were used. The assay technique with *L. citrovorum* was essentially that of Sauberlich and Baumann (1), with an acid-hydrolyzed casein substituted for the amino acid mixture of these workers. *S. faecalis* was used to assay the FA activity of CF concentrates; the basal medium and technique were those of Rabinowitz and Snell (12) with pyridoxal and cystine included in the basal medium but with FA omitted. Unless otherwise indicated, the cultures were incubated for 20 hours at 37°. The term "FA activity" as used throughout this communication refers to biological activity for *S. faecalis*, expressed in micrograms equivalence of FA.
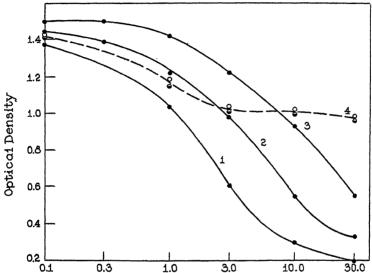
Experiments with Mice—The procedure used was that described by Franklin *et al.* (10) who showed that levels of 1 to 3 mg. of 4-amino-PGA per kilo of diet were lethal to mice in 1 to 2 weeks even when FA was added at levels up to 100 mg. per kilo of a purified diet. Carworth Farms, CF-1 strain, white mice, weighing 18 to 20 gm., were placed on a purified diet without FA containing 1 per cent succinylsulfathiazole. The effects of various supplements were studied by weighing the animals daily and observing them for diarrhea.

Experiments with Chicks—Day-old New Hampshire chicks were placed upon a purified diet as described previously (13) but with FA omitted. Vitamin B12, 0.5 γ per chick, was administered weekly by intramuscular injection. Under these conditions the chicks grew slowly and developed a mild anemia. Addition of FA to the diet enabled the chicks to grow at an approximately normal rate and corrected the anemia.

Results

Microbiological Studies with CF—Sauberlich showed (7) that CF concentrates from liver and rat urine were effective in reversing the growth inhibition of *L. citrovorum* by 4-amino-PGA. The data in Fig. 1 confirm these findings and demonstrate that CF reverses the inhibitory effect of 4-amino-PGA in a competitive manner. For example (Fig. 1, Curve 1), 10 CF units produced an optical density of 1.0 in the presence of 1 γ of

4-amino-PGA. When the CF concentration was increased to 30 units (Curve 2), 3 γ of 4-amino-PGA were required to limit growth to the same level (optical density 1.0). Similarly when the CF concentration was raised to 100 units (Curve 3), 10 γ of 4-amino-PGA were required for comparable growth inhibition. The observation that CF overcame the inhibitory effect of a structural analogue of FA in a competitive manner strongly suggested that CF is chemically related to FA.

FIG. 1. Effect of *citrovorum* factor (CF) and thymidine on inhibition of growth of *L. citrovorum* by 4-aminopteroylglutamic acid. Curve 1, 10 units of CF per 2 ml. of medium; Curve 2, 30 units of CF; Curve 3, 100 units of CF; Curve 4, 10 units of CF plus 3 γ of thymidine (O) or 10 γ of thymidine (●).

Several workers (1, 14) have demonstrated that thymidine partially replaces the unknown factor required by *L. citrovorum* and that thymidine will reverse the inhibitory effect of 4-amino-PGA for *L. citrovorum* (7) and *Escherichia coli* (8). When thymidine and CF were both present in the medium, 4-amino-PGA was markedly less inhibitory, as is shown by comparing Curves 1 and 4, Fig. 1. Moreover, the extent of inhibition by 4-amino-PGA was not altered whether 3 or 10 γ of thymidine were added per tube (Curve 4). Results obtained with thymidine alone (7) also indicated that this compound is a non-competitive antagonist of 4-amino-PGA. It may be postulated that the inhibitory effect of 4-amino-PGA on *L. citrovorum* is due to the blocking of reactions catalyzed by

CF and that thymidine is the end-product of such a reaction, thus accounting for the "non-competitive" reversal of 4-amino-PGA by thymidine.

In another experiment, the addition of hypoxanthine desoxyriboside, 3 γ, or FA, 1 to 10 γ per tube, was found to be without effect in modifying the response of the organism to CF in the presence of 4-amino-PGA.

If thymidine is a product of the action of CF, it might be expected that in the presence of thymidine the requirement of *L. citrovorum* for CF would be reduced. The curves in Fig. 2 demonstrate that, although
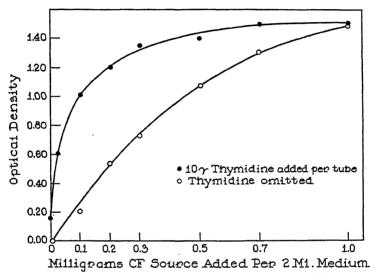
FIG. 2. The sparing action of thymidine on the requirement of *L. citrovorum* for CF. The CF source used was a papain digest of the coagulum obtained by cooking ground liver. The incubation period was 16 hours. Under these conditions thymidine does not produce appreciable growth of *L. citrovorum* (4).

thymidine by itself is ineffective in promoting growth, it significantly reduces the requirement of *L. citrovorum* for CF. These data are in agreement with a recent statement (6) that a synergistic action exists between thymidine and a factor active for *L. citrovorum*.

CF and 4-Amino-PGA Toxicity in Mice—Studies by Franklin and coworkers (10) indicated that 4-amino-PGA when fed to mice at a level of 1 part per million of diet resulted in death within a few days. Of particular interest was the fact that this effect was not reversed by feeding high levels of FA.

Table I summarizes the effect of FA and CF on the toxic action of 4-

amino-PGA for growth and survival of mice. Animals receiving 6 γ of 4-amino PGA injected three times weekly, with or without FA, lost weight very rapidly and after 5 days the mortality rate was very high. However, Group 8, receiving 6 γ of 4-amino-PGA and 200,000 units of CF, maintained their body weight throughout the test period and all the animals survived. Similar results were noted with higher levels of 4-amino-PGA, except that 200,000 units of CF afforded only partial protection against

TABLE I

Effect of Folic Acid (FA), Citrovorum Factor (CF), and 4-Aminopteroylglutamic Acid on Growth and Survival of Mice on Purified Diet Containing No Added FA

Twelve mice were used per group.

Group No.	Supplements injected per mouse 3 times weekly	Weight and No. of mice surviving (in parentheses)				
		1 day	3 days	5 days	7 days	9 days
		gm.	gm.	gm.	gm.	gm.
1	None	19.3 (12)	19.0 (12)	20.2 (12)	22.3 (12)	23.0 (12)
2	6 γ 4-amino-PGA	19.3 (12)	18.6 (12)	17.1 (12)	15.8 (5)	16.0 (3)
3	10 " "	19.5 (12)	18.2 (12)	17.3 (3)	14.0 (1)	0 (0)
4	20 " "	20.0 (12)	18.1 (12)	16.3 (3)	(0)	(0)
5	6 " " + 20 γ FA	18.8 (12)	18.0 (12)	15.4 (6)	14.0 (1)	(0)
6	10 γ 4-amino-PGA + 20 γ FA	18.0 (12)	18.5 (12)	16.2 (4)	14.0 (1)	(0)
7	20 γ 4-amino-PGA + 20 γ FA	19.2 (12)	17.8 (12)	15.0 (5)	13.0 (1)	(0)
8	6 γ 4-amino-PGA + 0.5 ml. CF*	19.3 (12)	19.5 (12)	20.1 (12)	19.1 (12)	19.5 (12)
9	10 γ 4-amino-PGA + 0.5 ml. CF	19.2 (12)	19.2 (12)	20.5 (12)	20.1 (12)	19.5 (12)
10	20 γ 4-amino-PGA + 0.5 ml. CF	19.3 (12)	18.9 (11)	18.0 (8)	15.8 (5)	15.7 (3)

* The CF concentrate contained 200,000 CF units per ml. and FA activity equivalent to 20 γ.

the 20 γ level of 4-amino-PGA. These data provide striking evidence of the marked biological activity of CF and imply that CF may be an important functional derivative of FA in metabolism.

It was also found that CF could protect against the toxicity of 4-amino-PGA when the two substances were incorporated in the diet (Table II). The results shown in Tables I and II indicate that for mice, as for *L. citrovorum*, CF reverses the effects of 4-amino-PGA in a competitive manner.

Chemical Properties of Citrovorum Factor—The Craig counter-current distribution procedure (15) was used in an attempt to concentrate CF, with

n-butanol and water at pH 2.0 as a solvent pair in which CF was found to have a distribution coefficient of approximately 1. In a typical experiment, 5 ml. of a solution containing 1,710,000 CF units and FA activity equivalent to 110 γ by *S. faecalis* assay were fractionated in ten tubes and the activity in each tube was determined microbiologically. The results, shown in Fig. 3, indicate that the distribution curves of CF and FA activity were indistinguishable within the limits of the assay methods.

The distribution coefficient of FA between butanol and water at pH 2.0 was determined spectrophotometrically and was found to be 1.2. This was considered to be additional evidence for a similarity between FA and CF. The data shown in Fig. 3 also suggested the possibility that CF can function as a replacement for FA in the nutrition of *S. faecalis*.

TABLE II

Reversal of 4-Aminopteroylglutamic Acid Toxicity in Mice by Incorporation of CF in Purified Diet

Twelve mice were used per group.

Group No.	Supplement added per kilo diet		Weight and No. of mice surviving (in parentheses)			
			1 day	5 days	9 days	13 days
			gm.	gm.	gm.	gm.
1	None		18.4 (12)	20.1 (12)	22.3 (12)	24.9 (12)
2	1 mg. 4-amino-PGA		18.2 (12)	18.2 (12)	15.5 (6)	14.5 (4)
3	2 " "		18.5 (12)	16.2 (12)	14.5 (2)	12.0 (1)
4	1 " "	+ 200 ml. CF*	18.8 (12)	20.1 (12)	22.2 (12)	24.6 (12)
5	2 " "	+ 200 " "	19.1 (12)	19.1 (12)	18.3 (12)	17.7 (12)

* The CF concentrate contained 30,000 CF units per ml. and FA activity equivalent to 19 γ.

In other similar counter-current experiments it was noted that marked losses in CF activity occurred during the manipulations. This was found to be due to the extreme lability of CF when standing in dilute acid at room temperatures. In contrast, the FA activity of the solutions was unaffected. These findings, shown in Table III, suggested that CF is an FA-like compound which can replace FA as a growth essential for *S faecalis*, and furthermore that upon standing in acid CF was decomposed, losing its activity for *L. citrovorum* and liberating a compound with FA activity for *S. faecalis*. These possibilities were investigated by chromatography. It was found that a column of Florisil (60 to 100 mesh) would adsorb CF but not FA from aqueous solutions at pH 4.0. A CF concentrate containing 100 γ of FA activity was mixed with a solution containing 100 γ of crystalline FA and the mixture was subjected to chro-

matography with the result that 95 per cent of the CF was retained on the column but only 50 per cent of the total FA activity of the mixture was found in the percolate. The next experiment was similar, except that the mixture was exposed to acid treatment before chromatography.
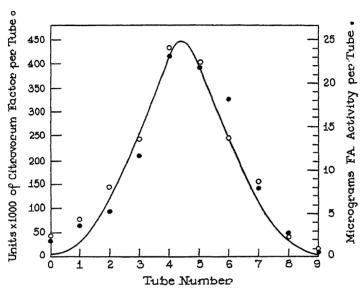
FIG. 3. Distribution of CF activity and folic acid activity in a liver fraction between *n*-butanol and water at pH 2.0. The curve represents the theoretical distribution curve of a substance having a distribution coefficient of 1; CF activity, O; folic acid activity, ●.

TABLE III

Effect of pH on Biological Activity of CF Concentrate

The solutions were assayed after standing for 30 hours at 25°.

pH of solution	CF content by *L. citrovorum* assay	FA content by *S. faecalis* assay, in terms of FA equivalence
	units per ml.	*γ per ml.*
8.0	165,000	44
2.0	3,300	46

As a result of this procedure the CF was destroyed and all the FA activity appeared in the percolate. These findings, summarized in Table IV, demonstrated that CF could replace FA in the nutrition of *S. faecalis* and that by treatment of CF with acid a compound with FA activity was liberated.

Effects of CF and 4-Amino-PGA upon S. faecalis—Both CF and FA were found to reverse the inhibitory effects of 4-amino-PGA for *S. faecalis*. Since *S. faecalis*, unlike *L. citrovorum*, can utilize either CF or FA for growth, it was possible to compare the effects of these factors in reversing 4-amino-PGA. The results, in Table V, indicate that CF was more

TABLE IV

Evidence for Liberation of FA-Like Compound from CF upon Treatment with Acid

Description of experiment	Per cent of orig-inal activity by *L. citrovorum* assay	FA content in terms of FA equivalence
		γ per ml.
1. To CF concentrate containing 1.1 million CF units and 100 γ FA activity, 100 γ FA added	100	189
2. Chromatographed (1) at pH 4.0 on Florisil column, washed well with water; percolate assayed	5.0	97
3. To CF concentrate containing 1.1 million CF units and 100 γ FA activity, 100 γ FA added and let stand at pH 2.0 for 30 hrs. at 25°	0.23	170
4. Chromatographed (3) at pH 4.0 on Florisil column, washed well with water; percolate assayed	0.12	176

TABLE V

Comparative Effects of CF and FA in Reversing Inhibitory Effects of 4-Amino-PGA for S. faecalis Grown on FA-Deficient Medium

4-Amino-PGA	Growth (optical density)	
	With PGA*	With CF†
γ per tube		
None	0.94	0.98
0.1	0.14	0.93
1.0	0.015	0.54
10.0	0	0.09

* 100 mμgm. per tube.
† 1000 units per tube, equivalent to 100 mμgm. of FA by *S. faecalis* assay.

effective than FA in reversing the antagonist. These findings further illustrate the interchangeable rôle of CF and FA in the nutrition of *S. faecalis*.

Results with Chicks—As a result of the preceding experiments, it was possible by comparative assays with *L. citrovorum* and *S. faecalis* to calculate approximately the respective amounts of CF and free FA in concentrates, for in the present results 1 unit of CF had FA activity corres-

ponding to about 0.1 mμgm. of FA.   In an experiment with chicks, a preparation was used which contained 20,000 CF units and 2.3 γ of FA activity per ml., thus indicating that most of the FA activity was present as CF.   The supplements were added to the diets since the experiments with mice had indicated that CF was utilized when administered in this manner.   The results, shown in Table VI, indicated that CF was at least as effective as FA in promoting the growth of chicks when added to a purified diet deficient in FA.

TABLE VI

Response of Chicks to Various Supplements When Added to Basal Purified Diet Deficient in FA

Ten to fourteen chicks were used per group.

Group No.	Addition per kilo basal diet	Average weights at		
		1 day	21 days	28 days
		gm.	gm.	gm.
1	None	42	100	107
2	1,000,000 units CF*	42	157	280
3	0.2 mg. FA	42	163	230
4	2.0 " "	42	177	281

* Concentrate described in the text.

DISCUSSION

In two recent notes, Bardos, Bond, Humphreys, Sibley, and Shive (5, 6) have enumerated some characteristics of "folinic acid," which appears to have properties corresponding with those of CF.   These workers stated that the potency for *L. citrovorum* of a concentrate of "folinic acid" was increased 5- to 10-fold by the addition of thymidine and that very mild acid hydrolysis destroyed "folinic acid" but formed a compound with biological activities corresponding to those of folic acid.   "Folinic acid" appeared to be not less active than folic acid in promoting the growth of either *L. casei* or *S. faecalis*.   The experiments described in the present communication led to similar conclusions and had been completed when these two notes appeared.   The finding that thymidine lowered the requirement of *L. citrovorum* is of importance in assaying for CF, as in natural materials containing thymidine the CF assay will yield erroneously high results unless CF is first separated from thymidine.   This has been shown to be possible by means of paper chromatography (4).

The conclusion that 1 CF unit represents about 0.1 mμgm. of FA enables a reevaluation of certain data to be made.   Sauberlich (9) found that the ingestion of supplementary FA markedly increased the urinary excre-

tion of CF; for example, a human subject excreted 425,000 units of CF following the administration of 30 mg. of FA. This would represent a conversion of only 0.04 mg. of FA into excreted CF. This apparently low rate of conversion of FA into CF *in vivo* is paralleled by the very slight effect of FA in reversing the toxicity of marginal levels of 4-amino-PGA in mice and the ineffectiveness of FA against higher levels of this antagonist (10). The suggestion by Sauberlich (9) that "part of the apparent disappearance of folic acid" (measured by assay with *S. faecalis*) "may be accounted for in the urine as the factor active for the growth of *L. citrovorum*" is obviated by the finding that CF will replace FA for *S. faecalis*.

Attention was recently drawn (16) to a probable rôle for CF in catalyzing the production of thymidine from certain other desoxyribosides. According to the present findings, this reaction would appear to be blocked by 4-amino-PGA, thus accounting for the respectively competitive and non-competitive actions of both CF and thymidine in reversing the inhibitory effects of 4-amino-PGA for *L. citrovorum*.

SUMMARY

1. Concentrates of a growth factor (CF) for *Leuconostoc citrovorum* 8081 were prepared from liver and were found to reverse in a competitive manner the inhibitory effects of 4-aminopteroylglutamic acid in experiments with *L. citrovorum*, *Streptococcus faecalis*, and mice.

2. Thymidine was found to reduce the requirement of *L. citrovorum* for CF.

3. Folic acid (FA) and CF were found to have similar distribution coefficients in the system *n*-butanol-water at pH 2.0.

4. CF was found to be destroyed at 25° in dilute acid solutions. Simultaneously a compound with FA activity was liberated. Both CF and the liberated compound were active as sources of FA for *S. faecalis*.

5. A concentrate of CF was found to be as effective as FA in promoting the growth of chicks on a purified diet from which FA was omitted.

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THERMAL COAGULATION OF SERUM PROTEINS

III. THE EFFECTS OF pH AND OF SULFHYDRYL REAGENTS ON THE NATURE OF THE COAGULUM*

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In solutions of serum heated at 100° at neutrality, the presence of small amounts of sulfhydryl reagents markedly alters the course of coagulation; iodoacetic acid strongly inhibits the formation of a coagulum, while iodoacetamide, methyl iodoacetate, and *p*-chloromercuribenzoate accelerate the rate at which a solid gel is obtained (1). The formation of a solid one-phase coagulum or gel in a heat-denatured serum protein solution requires a certain minimum protein concentration, and the determination of this "least coagulable concentration" can serve as a measure of the coagulative ability of a serum or protein solution (2).

The present paper describes the thermal coagulation of bovine plasma albumin and human serum albumin, showing that in each case two different types of coagula can be produced. The nature of the coagulum, as well as the protein concentration required to form a clot or gel, depends on the pH of the system and is also markedly influenced by exceedingly minute amounts of compounds which can react with sulfhydryl groups.

EXPERIMENTAL

Materials

Crystallized bovine plasma albumin (Armour) was used in the majority of the experiments. Solutions were prepared fresh daily from the dry material. Since this preparation usually contains about 10 per cent moisture, the actual albumin content of the solutions was determined by Kjeldahl nitrogen analysis; unless otherwise noted, the values given in this paper refer to weights of anhydrous protein.

Human albumin was prepared by the fractionation of normal human serum by the method of Cohn *et al.* (3); this substance was electrophoretically homogeneous at pH 8.6.

Acetylated bovine plasma albumin was prepared by the method of Hughes as described by Olcott and Fraenkel-Conrat (4). Analysis indicated that 99 per cent of the free amino groups and 92 per cent of the sulfhydryl groups were acetylated. This material was soluble in water and buffer solutions, if the pH was 5.5 or higher, and was reversibly precipitated when the pH was lowered below that point.

Methods

In the determination of the least coagulable concentration of a protein, a series of tubes (13 × 100 mm.) was set up containing varying amounts of a solution of protein in buffer, and the final volume in each was made to 2.0 ml. with buffer. With bovine albumin, the concentration was varied in 1.0 mg. per ml. intervals, e.g. 11, 12, 13 mg. per ml. With human albumin, the intervals were 0.5 mg. per ml., e.g. 5.0, 5.5, 6.0 mg. per ml. The range of concentrations was chosen on the basis of preliminary investigation so that the more concentrated solutions of each series would form a solid gel when heated, while the least concentrated would remain liquid. The tubes were immersed in a stirred water bath at 99–100° for 30 minutes, after which they were cooled for 5 minutes in tap water. A loop of fine stiff wire (No. 8 piano wire) fitting tightly against the inside of the tube was then passed gently to the bottom of the tube to loosen the clot from the glass, and the contents of the tube were allowed to slide gently from the tube to a clean, dry, stainless steel wire screen (8 mesh, wire diameter 0.7 mm.). If an appreciable amount of the contents passed through the screen within 1 minute, the system was considered as liquid, otherwise as a gel. The smallest concentration of protein to form a gel in each series was designated as the least coagulable concentration. The results were reproducible within about 3 per cent.

The effect of pH on the thermal coagulation of proteins was investigated in 0.1 M acetate buffers over the pH range 4.5 to 5.8 and in 0.1 M phosphate buffers over the range 5.8 to 9.4. The pH of each buffer mixture was determined with a glass electrode at 90–95°, and the values given in this paper refer to that temperature.

The effect of various reagents upon the least coagulable concentration at pH 6.9 and 7.4 was studied by preparing solutions of protein in phosphate buffer with the reagent in the desired molar proportion. In the case of reagents which react with phosphate (e.g. Ag^+, Cu^{++}, etc.), the protein and reagent were mixed in a minimum of distilled water before dilution with buffer. Solutions thus prepared do not deposit phosphate at the lower metal-protein ratios.

To determine the water-binding properties of a coagulum, small pieces were placed on a fine screen (50 mesh) and allowed to soak in water over-

night at room temperature. The excess water was then removed from the coagulum by dipping the system gently in acetone and twice in ether. After being dried for a few minutes in a gentle air stream to remove the ether, the moist coagulum was weighed. The material was then dried at 110° for 2 hours and the dry protein residue weighed again. The bound water is the difference between these two weights and is expressed as mg. of water per mg. of dry protein residue.
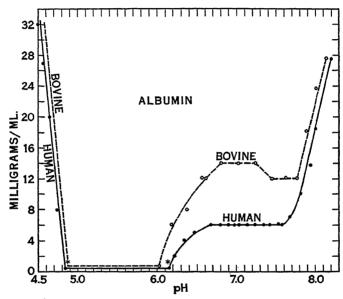
FIG. 1. Least coagulable concentrations of albumin solutions at different pH conditions. The ordinates should be multiplied by 0.91 to express the results as anhydrous protein. Opaque two-phase coagulum formed between pH 4.8 and 6.0; solid gels with decreasing turbidity from pH 4.9 to 4.5 and from pH 6.1 to 8.0.

Determination of amino groups was carried out by the method of Van Slyke (5) as modified by Kendrick and Hanke (6), with a reaction time of 15 minutes. When a protein coagulum was analyzed, it was first homogenized with 2 parts of water in a Potter-Elvehjem glass homogenizer. Sulfhydryl groups were determined by amperometric titration (7).

Results

Effect of pH on Coagulation—As the pH of the system is varied, serum albumin shows two distinct types of coagulation (Fig. 1). In the region pH 4.8 to 6.0, coagulation is rapid with turbidity forming within 30 seconds at 100°. Concentrations as low as 0.3 mg. per ml. produced aggregates;

with 3 to 30 mg. per ml. dense white coagula formed which underwent marked syneresis when touched with the wire loop. Since coagula of this kind consist of a solid and liquid phase, the least coagulable concentration as defined has no application to this pH range.

At pH values lower than 4.8 and higher than 6.0, the coagulum is monophasic, and progressively increasing protein concentrations are required for the formation of solid gels. As the pH is lowered from 4.8, the least coagulable concentration increases very rapidly and the turbidity of the clot decreases until at pH 4.2 the gel is water-clear. On progressing upward from pH 6.0 the least coagulable concentration rises slowly, levels off in the region of pH 6.8 to 7.7, and then rises again, the turbidity decreasing until at pH 9.0 the gels are clear.[1] As the turbidity of the coagulum becomes less, the tendency for syneresis to occur on standing is markedly decreased.

Compared to the formation of the turbid coagulum, which can occur within 30 seconds at pH 5.5, the production of a solid gel at higher pH values is a relatively slow process, usually occurring in 8 to 15 minutes at 100° depending on protein concentration. Bovine albumin solutions heated at 100° for 2 minutes at pH 7.4 were practically clear. Acidification of the cooled solutions to pH 5.5 formed a dense white precipitate, indicating that the protein was already denatured but that aggregation was inhibited under conditions of higher pH.

The two types of albumin coagula bear a certain resemblance to the two types of fibrin clots obtained by Ferry and Morrison (9) from the clotting of fibrinogen by thrombin at different pH conditions. The fibrin clot obtained at pH 6.3 was opaque and synerizing, while the clot formed at pH 8.5 was clear and non-synerizing.

Influence of Reagents on Gel Properties—In the pH range 6.9 to 7.4, thermal coagulation of albumin solutions results in a soft opaque gel (Fig. 2). If small amounts of certain reagents, which have in common the ability to react with sulfhydryl groups, are present during thermal coagulation, the properties of the coagulum are markedly altered. Gels thus formed are firm, elastic, practically clear, and do not show the syneresis which occurs on standing with the opaque gels. Reagents which at pH 6.9 to 7.4 promote the formation of firm clear clots, when present in amounts of 20 or fewer equivalents per mole of albumin, are iodoacetamide, iodine, iodosobenzoate, *p*-chloromercuribenzoate, chloropicrin, silver nitrate, cadmium nitrate, cupric nitrate, mercuric nitrate, hydrogen peroxide, and

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[1] In a recent study of the minimal temperature required for coagulation of rather concentrated solutions (90 mg. per ml.) of horse serum albumin, Barbu and Macheboeuf (8) observed a region of turbid coagulum formation at pH 5 to 6 bounded by regions of progressively clearer gel formation at higher or lower pH.

cumene hydroperoxide. In contrast to the other sulfhydryl reagents, sodium iodoacetate is a marked inhibitor of thermal coagulation; in its presence the heated protein solutions remained clear and liquid. No effect is noticeable with similar amounts of sodium chloride, sodium acetate, zinc nitrate, aluminum nitrate, ferric chloride, calcium nitrate, magnesium nitrate, potassium iodide, acetamide, or benzoyl peroxide. In the presence of cysteine, propyl mercaptan, or sodium cyanide, turbidity formation occurs more rapidly and the clot formed is more opaque and less rigid.

The clear clots bind water firmly within the gel network. This property is demonstrated qualitatively by increased rigidity and resistance to syn-
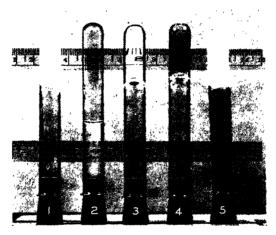
Fig. 2. Influence of reagents on nature of coagulum of bovine albumin. The tubes contain 64 mg. of bovine albumin in 3.0 ml. of phosphate buffer of pH 7.4 with addition of 20 μM of the following reagents: Tube 1 none (control), Tube 2 sodium iodoacetate, Tube 3 iodoacetamide, Tube 4 p-chloromercuribenzoate, Tube 5 cysteine hydrochloride. Tubes heated 20 minutes at 100° and inverted.

eresis. Estimates of the water bound by the different types of coagula are given in Table I. These data show that the clear gels, formed in the presence of reagents which react with the sulfhydryl group, can hold 3 to 4 times as much water as can the opaque gel formed from albumin alone. The amount of water bound per unit weight of albumin is independent of the protein concentration over the range 20 to 60 mg. per ml.

The effect of sulfhydryl reagents on the least coagulable protein concentration of bovine albumin at pH 6.9 is summarized in Fig. 3; the results obtained at pH 7.4 and from similar experiments with human serum albumin were analogous. The data indicate that these reagents strongly enhance gel formation; the protein concentration required for clotting is

Water Binding of Bovine Albumin Gels*

Added reagent	Reagent per mole protein	Water bound by protein
	equivalents	*mg. per mg.*
None (control)		13, 14, 16†
Cysteine·HCl	20.0	15, 16
Barium nitrate	2.6	15
Silver "	1.3	33
Iodine	2.6	36
Iodosobenzoate	1.3	35, 38
Iodoacetamide	20.0	46, 47
p-Chloromercuribenzoate	12.0	55, 59
Chloropicrin	3.0 *Ca.*	56

* Gels were formed by heating solutions of bovine albumin (21 mg. per ml. in M/15 phosphate buffer, pH 7.4) for 20 minutes at 100°.

† More than one value indicates repeated determinations.
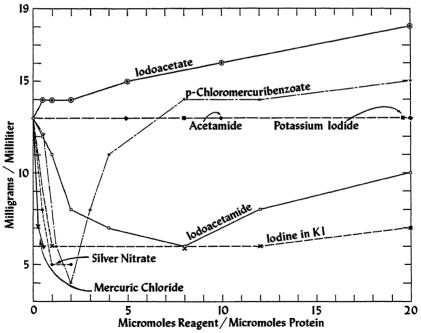
FIG. 3. Effects of reagents on least coagulable concentration of bovine albumin in 0.1 M phosphate buffer, pH 6.9.

reduced to less than one-half the control value. In most cases, 1 equivalent of reagent per mole of albumin produces the maximum effect; with

iodoacetamide 4 to 8 molar equivalents are required. The other sulf-hydryl reagents mentioned above behave similarly with the exception of cupric nitrate, which has little effect on the least coagulable concentration, and sodium iodoacetate which inhibits coagulation.

Although the gels formed in the presence of 1 molar proportion of reagent are much less opaque than those of albumin alone, maximum clarity is usually reached in the presence of 4 to 5 equivalents of reagent per mole of protein, more reagent being required at pH 6.9 than at pH 7.4. In the case of iodoacetamide, about 10 equivalents are required for maximum clarity.

In contrast to this striking effect at pH 6.9 to 7.4, sulfhydryl reagents show no appreciable effect on the opaque coagulum which forms rapidly at pH 5.5.

Relation of Amino Groups to Thermal Coagulation—By our measurements, native bovine plasma albumin contains 9.0 to 9.1 moles of amino groups per 10^4 gm. corresponding to 62 to 63 amino groups per albumin molecule.[2] When solutions of bovine albumin at pH 7.4 are heated for 20 minutes at 100°, there is a slight increase in the number of amino groups which react with nitrous acid to liberate nitrogen; heated albumin solutions show 66 amino groups per molecule. This is true either if a solid gel is formed or if the solution is so dilute that no clotting occurs. Thus it appears that in thermal coagulation the amino groups of albumin do not participate in aggregation by the formation of covalent bonds.

However, acetylation of the amino groups of albumin imparts a marked resistance to thermal coagulation. At pH 6.5 the least coagulable concentration of acetylated bovine albumin is 70 mg. per ml., as compared with 10 mg. per ml. for the untreated protein. Moreover, coagula of acetylated albumin are perfectly clear; no trace of turbidity is present either in the liquid formed with lower protein concentrations or the gels formed with higher protein concentrations.

<div align="center">DISCUSSION</div>

The thermal denaturation of a protein such as albumin is believed to involve breaking of hydrogen bonds which hold the native protein in its specific configuration, causing an unfolding and extension of the molecule (14). If the concentration of protein in the solution is sufficient, visible aggregation of the denatured protein molecules occurs; it has been suggested that the molecules in the coagulum are linked together through new hydro-

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[2] These figures are intermediate between the results of Olcott and Fraenkel-Conrat (4) and the calculations of Brand (10) indicating respectively 59 and 68 amino groups per molecule of bovine albumin (molecule weight 69,000). The amino group content of a molecule of native human serum albumin has been reported as 60 (11), 61 to 68 (12), 67 (10), and 64 to 68 (13).

gen bonds formed between carboxyl and amino groups of neighboring polypeptide chains.

A gel is usually considered to involve a cross-linked three-dimensional network of long molecules which is capable of binding large amounts of solvent within the network. Clear fibrin clots have been postulated to consist of a regular network of polypeptide chains linked together at relatively few points on the molecule with many interstitial spaces in which water can be bound to the protein (9). Opaque synerizing fibrin clots are considered to possess additional association at many sites along the molecule; thus the polypeptide chains lie close together in heterogeneous clumps which scatter light and have relatively few interstitial spaces for binding water. Subsequent electron microscopic investigation of these fibrin clots has in a general way supported this concept of gel structure (15).

The properties of the two types of albumin gels suggest that there are two types of aggregation which link the denatured albumin molecules in the coagulum, a three-dimensional gel network in the clear clots plus additional lateral association in the opaque clots. In the region of the isoelectric point, intermolecular repulsive forces are minimal; thus side by side aggregation of the denatured protein molecules rapidly occurs. As the pH is raised or lowered from the isoelectric region, there is a progressive increase in the net charge (negative or positive respectively) of the protein with a corresponding increase in the intermolecular repulsion. Both network formation and lateral association are progressively inhibited as the net charge on the denatured protein increases. The effect of this repulsion on gel network formation is evident from the increased protein concentration required to form a solid gel (Fig. 1); the effect on side by side association is shown by the rapid decrease in the turbidity of the gels as the pH becomes further removed from the isoelectric point. Intermolecular repulsion apparently has a greater inhibitory effect on lateral association than it has on network formation, since the gels formed pass from the soft opaque to the clear firm type of clot as these forces increase.

Since the formation of the coagulum depends on the aggregation of denatured albumin molecules, the pH region of minimal repulsion should be determined by the isoelectric point of denatured rather than native albumin. The isoelectric point of a denatured protein lies between that of the native protein and neutrality (14), and for denatured serum albumin has been found to occur between pH 5.1 and 5.3 (16); this point is fairly close to the center of the region of minimal repulsion in Fig. 1.

If this concept of the processes operating during the aggregation of thermally denatured albumin is valid, it should be possible to attach some significance to the shape of the curves in Fig. 1. The steepness of the curves at pH values lower than 4.8 indicates that repulsive forces increase very rapidly with decreasing pH, reflecting a rapid increase in the net posi-

tive charge of the protein molecule. On the other hand, the fact that, at pH values above the isoelectric region, the least coagulable concentration increases rather slowly indicates that in this region repulsive forces, and presumably net negative charge, increase only slowly with pH. These conclusions agree with those derived from inspection of the acid-base titration curve of human serum albumin (11), if one assumes that the titration curve of denatured albumin is somewhat similar to that of the native protein. The plateaus in the curves of Fig. 1, which suggest that in this region the net charge of the denatured albumin molecule changes little with change in pH of the medium, occur at about the same pH as the point of inflection of the titration curve.

The nature of the linkages binding the polypeptide chains in a gel network and in lateral association is uncertain, but it is reasonable to assume that the close side by side aggregation is due to intermolecular hydrogen bonds, probably between side chain amino and carboxyl groups, as proposed by Mirsky and Pauling (14). The sensitivity of turbidity formation to changes in the net electrostatic charge is in agreement with the properties of hydrogen bonds, as these authors describe them.

From the foregoing considerations, conditions which inhibit lateral association of the polypeptide chains should decrease the turbidity of the coagulum. At high or low pH values, intermolecular repulsion due to net electrostatic charge accomplishes this effect. However, the net electrostatic charge of the protein may be increased without changing the pH. Acetylation of the free amino groups removes cationic centers and increases the net negative charge; the behavior of the acetylated albumin on coagulation at pH 6.5 to 7 resembles that of untreated albumin at pH 10. Sodium iodoacetate, by reaction with sulfhydryl and amino groups, introduces additional carboxyl anions into the protein molecule, which increases its net negative charge (1). Thus sodium iodoacetate inhibits both gel formation and turbidity, the inhibition becoming significant when 5 or more equivalents of iodoacetate are present (Fig. 3).

Elimination of turbidity by pH change, acetylation of amino groups, or addition of iodoacetate appears to involve inhibition of gel network formation as well as lateral association inasmuch as the least coagulable concentration under these conditions is markedly increased. If side by side aggregation can be prevented without the inhibition of network bond formation, clear gels should be produced with protein concentrations less than that of the control, since each polypeptide chain, when it is not drawn close to its neighbor, should be more efficient in building a network capable of enmeshing the whole of the solvent. Sulfhydryl reagents possess this property; they prevent turbidity in the coagulum and at the same time lower the least coagulable concentration.

Since serum albumin contains approximately one sulfhydryl group per

albumin molecule,[3] the maximal effect of sulfhydryl reagents on the least coagulable concentration in molar proportions of 1:1 strongly indicates that
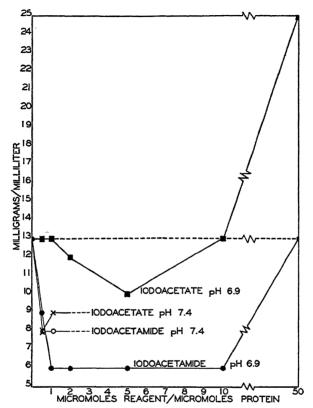
FIG. 4. Least coagulable concentration of bovine albumin solutions incubated with iodoacetate and iodoacetamide for 24 hours at 37°. In the experiments at pH 6.9 coagulation at 100° was carried out directly following incubation; at pH 7.4 the incubated solutions were first dialyzed and lyophylized, and the residues redissolved in buffer for coagulation studies.

the increased gel-forming action is directly related to reaction with the sulfhydryl group.   One is led to the conclusion that, in the pH region 6.9 to 7.4, the presence of one free sulfhydryl group per protein molecule pro-

[3] Human serum albumin crystallized with the aid of decanol has been shown to contain an average of 0.7 sulfhydryl group per albumin molecule (11). Amperometric titrations carried out in this laboratory indicate an average of 0.75 sulfhydryl groups per albumin molecule in normal human serum albumin (12) and 0.71 in crystallized bovine plasma albumin.

motes lateral association of the denatured albumin molecules, and that inactivation of this group, either by oxidation (I_2, H_2O_2, iodosobenzoate), alkylation (iodoacetamide, chloropicrin), or mercaptide formation (Ag^+, Cd^{++}), prevents side by side binding from occurring.

Under the usual experimental conditions, iodoacetamide and iodoacetate appear to differ from the other sulfhydryl reagents in that maximum lowering of the least coagulable concentration is not observed in the presence of 1 equivalent of reagent. This apparent anomaly is probably due to relative reaction rates. The reaction of iodoacetamide with sulfhydryl groups at room temperature and pH 6.9 is rather slow and that of iodoacetate even slower (17). At pH 7.4, reaction is somewhat faster but still slow compared to that of reagents such as iodine, silver ion, or p-chloromercuribenzoate. Whereas the latter substances probably react completely with the protein upon mixing, complete reaction of iodoacetate and iodoacetamide with the sulfhydryl group requires either a long reaction time or an excess of reagent. As previously discussed, excess iodoacetate inhibits coagulation, an effect which counteracts the gel enhancement resulting from blocking of the sulfhydryl group so that lowering of the least coagulable concentration is not observed at all. However, if a sufficient period is allowed prior to thermal coagulation so that reaction with the sulfhydryl group can proceed to completion, lowering of the least coagulable concentration by 1 equivalent of iodoacetamide or iodoacetate resembles that of the other sulfhydryl reagents (Fig. 4).

To exert its marked effect on thermal coagulation, the sulfhydryl group must occupy a key position on the peptide chain. It is interesting to consider how one group might promote lateral association of the whole molecule. In a pH region (6.9 to 7.4) in which repulsive forces may be sufficient to prevent participating groups from approaching close enough to form hydrogen bonds, the sulfhydryl group may be able to form a covalent linkage with some group of a neighboring peptide chain; the ability of sulfhydryl groups to effect cross-linking of synthetic polymer chains is well known. The presence of one firm bond between adjacent denatured protein molecules might then increase the probability of further lateral association by hydrogen bonds. In any case, the remarkable fact that one side group can profoundly influence the coagulation of a molecule containing nearly 600 amino acid residues implies unique properties for the group in question.

SUMMARY

1. Depending on the pH of the medium, heat-denatured solutions of serum albumin form opaque synerizing coagula, clear non-synerizing gels, or clots intermediate between these two types.

2. The nature of the coagulum and the minimum albumin concentration required for gel formation have been interpreted in terms of intermolecular repulsive forces due to the net electrostatic charge of the denatured albumin molecules.

3. The physical properties of the coagulum obtained from serum albumin at pH 6.9 to 7.4 depend in considerable measure on the presence of one free sulfhydryl group per protein molecule. If this sulfhydryl group is destroyed by chemical reagents, thermal coagulation is markedly altered in that the least coagulable concentration is lowered, water binding is increased, and clear clots result.

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ON THE RÔLE OF 2,6-DIAMINOPURINE IN THE BIOSYNTHESIS OF NUCLEIC ACID GUANINE*

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It has been demonstrated (1, 2) that dietary guanine is not utilized to a significant extent by the rat for nucleic acid synthesis but that adenine may be utilized by the rat as a precursor of both polynucleotide adenine and polynucleotide guanine (2). Recent studies with labeled glycine (3) and nucleic acid (4) have indicated that polynucleotide guanine may also arise by mechanisms not involving the mediation of adenine. The mechanism by which adenine is converted into polynucleotide guanine remains an open question and the present communication deals with a consideration of certain possible routes by which this transformation might occur. These are outlined in the scheme in Fig. 1.

The failure of isoguanine (III) (5) and of hypoxanthine (V) and xanthine (VI) (6) to serve as precursors of nucleic acid purines has already been noted. However, as was reported in a preliminary communication (7), 2,6-diaminopurine (IV) was found to be an effective precursor of nucleic acid guanine (II).[1]

The initial experiment was carried out with 2,6-diaminopurine labeled with heavy nitrogen. For the synthesis with N^{15}, isotopic ammonium nitrate was fused with dicyandiamide to yield guanidine nitrate (8), which was condensed with malononitrile to give 2,4,5,6-tetraaminopyrimidine after nitrosation and reduction (9). 2,6-Diaminopurine, containing excess N^{15} in the 1 and 3 positions as well as in the 2-amino group, was obtained by use of the formic acid-formamide method (10). The synthesis is outlined in Fig. 2.

[1] Several preliminary experiments have been carried out with *purineless* mutants of *Escherichia coli* which grow well when adenine, guanine, hypoxanthine, or xanthine is added to the cultures. 2,6-Diaminopurine was found to be inactive. Isoguanine, as well as its riboside (crotonoside), supported the growth of the mutants, although the growth was slower. Isoguanine, but not crotonoside, was found to be inhibitory at high concentrations (Davis, B., and Bendich, A., unpublished results).

This compound was given by stomach tube to adult rats in single daily doses of 29 mg. (0.2 mM) per kilo for 3 days. The nucleic acids isolated from the mixed organs were processed for adenine, guanine, and py-

FIG. 1

FIG. 2

rimidines. Various urinary constituents were isolated. The results are shown in Table I from which it can be seen that the guanine had been derived from the dietary 2,6-diaminopurine. It is calculated that about 4.0 per cent of the guanine of the combined nucleic acids had arisen from the dietary purine. This renewal is directly comparable to that in the

experiment in which adenine was administered at the same molar level for the same period of time (2), and in which it was found that about 3.2 per cent of the nucleic acid guanine had been derived from the dietary adenine. This is consistent with the proposition that 2,6-diaminopurine participates as an intermediate in the conversion of adenine to nucleic acid guanine in the rat.

The N^{15} values of the nucleic acid pyrimidines and the urinary urea and ammonia, 0.020 to 0.023 per cent excess N^{15}, are almost identical. It

TABLE I

Feeding of N^{15}-2,6-Diaminopurine

	Atom per cent excess N^{15} *	Calculated on basis of 100 per cent N^{15} in purine fed
2,6-Diaminopurine (dietary).........................	5.45	100
Sodium nucleic acids.................................	0.076	
Copper purines......................................	0.148	
Adenine (calculated from picrate)†..................	0.043	0.78
" (free)†.......................................	0.042	
Guanine sulfate.....................................	0.222	4.0
" (free)...	0.216	
Silver pyrimidines...................................	0.022	0.40
Total urinary nitrogen..............................	0.053	
Allantoin...	0.336	6.2
Urea...	0.023	0.42
Ammonia...	0.021	0.38

* Consolidated-Nier ratio mass spectrometer, model 21-201. The values are averages of duplicate determinations; probable error for N^{15} ±0.001, for C^{13} about ±0.005.

† The adenine contained a trace of foreign material with an R_F value of that of guanine when examined by paper chromatography.

should be pointed out that these values are of the same magnitude as the corresponding values obtained in several experiments carried out in this laboratory in which guanine, also labeled in the 2-amino group, had been administered in single daily doses by intraperitoneal injection.

The N^{15} content of the adenine, 0.042, is about twice the "base-line" value derived from ammonia in the body. This probably does not indicate any specific synthesis of adenine from the dietary 2,6-diaminopurine, for the adenine specimen was found to be contaminated with a small amount of material having properties (R_F values in paper chromatography) resembling guanine. 2,6-Diaminopurine could not be demonstrated in any of the fractions.

In order to determine with assurance whether there was any specific conversion of 2,6-diaminopurine into adenine, a second experiment was carried out. For this purpose 2,6-diaminopurine, containing excess C^{13} in the 2-carbon atom, was synthesized and fed. For the synthesis, potassium cyanide, containing an excess of C^{13}, was converted by bromination to cyanogen bromide (11). The isotopic cyanogen bromide was condensed with ammonia (10) and the resulting cyanamide was converted to guanidine nitrate upon reaction with aqueous ammonium nitrate (12). The synthesis is outlined in Fig. 3. 2,6-Diaminopurine, with excess C^{13}

FIG. 3

TABLE II

Feeding of 2-C^{13}-2,6-Diaminopurine

	Atom per cent excess C^{13} *	Calculated on basis of 100 per cent C^{13} in purine fed
2,6-Diaminopurine (dietary)	10.7	100
Sodium nucleic acids	0.000	
Nucleic acids	−0.001	
Purine hydrochlorides	0.049	
Adenine (free)	−0.003	0.00
Guanine sulfate	0.163	1.5
Allantoin	0.602	5.6
Guanidine picrate from guanine sulfate	0.098	
Calculated for guanidine picrate from position 2 only	0.115	

* See foot-note to Table I.

in carbon 2, was obtained from the isotopic guanidine in the manner described above.

This preparation was fed to rats at a level of 29 mg. per kilo per day for 3 days. The adenine and guanine isolated from the nucleic acids were found to be homogeneous and free from contamination. The results (Table II) show unambiguously that there was no incorporation of isotope into the adenine, whereas there was extensive synthesis of guanine from the dietary 2,6-diaminopurine. During the course of the experimental period, about 1.5 per cent of the nucleic acid guanine had been synthesized from the dietary 2,6-diaminopurine. This figure is considerably lower than that (4.0) for the parallel N^{15} experiment. The explanation for this difference is not apparent.

In order to determine whether the 2-carbon atom had remained in the same position in the ring system throughout the conversion of dietary 2,6-diaminopurine to the guanine incorporated into the nucleic acids, a sample of the isolated guanine was oxidized to guanidine by means of chloric acid (2). From the values cited in Table II it is seen that, so far as this carbon atom is concerned, there was an extensive retention of the ring system during the conversion.

Since it is known (13) that in the adult rat the incorporation of adenine into the desoxypentose nucleic acid (DNA) fraction is only about 1 per cent of that into the pentose nucleic acid (PNA) fraction, these data do not serve to indicate whether 2,6-diaminopurine can serve as a precursor

TABLE III

Feeding of Isotopic 2,6-Diaminopurine to Partially Hepatectomized Rats

	Atom per cent excess N^{15} *	Calculated on basis of 100 per cent N^{15} in purine fed
2,6-Diaminopurine (dietary).........................	5.45	100
Pentose nucleic acids		
Copper purines....................................	0.132	
Adenine...	0.015	0.28
Guanine..	0.202	3.7
Desoxypentose nucleic acids		
Copper purines....................................	0.089	
Adenine...	0.011	0.20
Guanine..	0.198	3.6

* See foot-note to Table I.

of DNA guanine as well as of PNA guanine. Subtotal hepatectomies were therefore performed on several rats and 2,6-diaminopurine (labeled with excess N^{15}) was administered by stomach tube for 5 days beginning on the 1st postoperative day. It was administered at half the level (14.5 mg., 0.1 mM per kilo per day) because of the somewhat greater toxicity in hepatectomized animals. The livers which were removed on the 6th day showed the expected regeneration. From the PNA and DNA fractions prepared from the livers, guanine and adenine were isolated. The results of this experiment, given in Table III, reveal that, under these conditions of DNA synthesis, 2,6-diaminopurine can serve as an effective precursor of DNA, as well as of PNA, guanine. It is of interest to note that the extent of guanine renewal was very nearly the same in both nucleic acids and that the dietary 2,6-diaminopurine was not utilized for adenine synthesis. The small amounts of N^{15} found in the adenine, 0.011 to 0.015

atom per cent, undoubtedly represent synthesis from breakdown product which have contributed to the ammonia in the body.

Isotopic 2,6-Diaminopurine. Labeled with N^{15} in 1 and 3 Positions an in 2-Amino Group—Guanidine nitrate was prepared (8) from dicyandia mide and ammonium nitrate (containing about 32 atom per cent exces N^{15} in the ammonium radical). The isotopic guanidine nitrate was con densed with malononitrile and the product was converted after nitro sation and reduction to 2,4,5,6-tetraaminopyrimidine sulfate according t(a procedure described previously (9). The tetraaminopyrimidine was con verted to 2,6-diaminopurine, in 88 per cent yield, by the formic acid formamide method (10). The compound was recrystallized from 0.2 N H_2SO_4 and was dried at room temperature over P_2O_5 in a vacuum desiccator.

$C_5H_6N_6 \cdot \frac{1}{2}H_2SO_4 \cdot \frac{1}{2}H_2O$. Calculated, N 38.7; found, N 38.7

The spectrum was that of 2,6-diaminopurine (14). The preparation was homogeneous when examined by counter-current distribution, with distribution coefficient $K = 1.16$ in *n*-butanol-1 M phosphate, pH 6.57 (14). The product contained 5.45 atom per cent excess N^{15}.

Labeled with C^{13} in 2-Carbon—A solution of 10.5 gm. of potassium cyanide (containing 54 per cent excess C^{13}) in 48 ml. of water was slowly added to a solution of 6.4 ml. of bromine and 2 ml. of water that was continuously stirred and kept below 30°. The mixture was carefully distilled at 58–68° on a water bath and about 9 gm. of product were collected (11). After drying over P_2O_5, the product was redistilled and 8.26 gm. of isotopic cyanogen bromide which boiled at 58–62° were obtained. This was converted to cyanamide by the following procedure (10).

Ammonia, generated from 12.4 gm. of NH_4NO_3 in 30 ml. of water by means of 18 ml. of 12 N NaOH, was dried by passage through a condenser and through a drying tower filled with NaOH pellets and was condensed in a tube containing 65 ml. of absolute ethanol cooled in a dry ice-alcohol bath. The isotopic cyanogen bromide (8.26 gm.) in 50 ml. of dry ether was added to the cold alcoholic ammonia solution. The tube was securely stoppered and was kept overnight at room temperature. The NH_4Br which had formed (6.91 gm.) was collected and the filtrate was concentrated to dryness *in vacuo*. Ether (50 ml.) was added to the residue and an additional 0.44 gm. of NH_4Br was removed by filtration. The filtrate was taken to dryness *in vacuo* and yielded 2.94 gm. of cyanamide (90 per cent yield).

Isotopic cyanamide (1.65 gm.) was dissolved in 10 ml. of a solution con-

taining 4.72 gm. of NH_4NO_3 and the solution was heated in a bomb tube at 155° for 3 hours (12). After chilling, 1.96 gm. of guanidine nitrate (m.p. 213–214°) were collected and an additional 1.20 gm. were obtained from the mother liquors (66 per cent yield).

The 2,6-diaminopurine sulfate (with excess C^{13} in the 2-carbon atom), prepared from the isotopic guanidine nitrate by the above procedure, contained 10.7 atom per cent excess C^{13}. The spectrum, determined in 0.1 M phosphate buffer, pH 6.5, showed ϵ_M (280 mμ) = 7280.

$C_5H_6N_6 \cdot \frac{1}{2}H_2SO_4 \cdot \frac{1}{2}H_2O$. Calculated, N 38.7; found, N 38.7

Feeding Experiments. N^{15}-*2,6-Diaminopurine*—A 0.4 per cent suspension of 2,6-diaminopurine sulfate in 10 per cent gum acacia was homogenized and administered in single doses at a level equivalent to 29 mg. of free base per kilo per day by stomach tube to three male rats of the Sherman strain for 3 days. The rats weighed a total of 618 gm. and were kept on their usual diet of Purina animal chow. The urinary output was collected and pooled. The animals were sacrificed on the 4th day and the testes and various organs were frozen in dry ice-alcohol, homogenized, and dried. Total nucleic acids were prepared from the dried organs and were processed for copper purines, adenine, guanine, and pyrimidines by procedures reported previously (2). The guanine was found to be homogeneous by means of paper chromatography (15), but the adenine fraction contained a trace of foreign material with R_F values of that of guanine. Allantoin, urea, ammonia, and total N^{15} of the urine were also determined (2). The results are given in Table I.

C^{13}-*2,6-Diaminopurine*—A suspension of C^{13}-labeled 2,6-diaminopurine sulfate was made up as in the previous experiment and was administered for 3 days at the same level (29 mg. of free base per kilo) to five male rats with an aggregate weight of 1.79 kilos. The animals were sacrificed on the 4th day and were worked up as in the previous experiment with the following minor changes. The sodium nucleate was converted to free nucleic acid, the purine hydrochlorides were prepared, and adenine picrate and guanine sulfate were obtained. The adenine picrate was converted to free adenine. The identity and homogeneity of these compounds as well as of guanine sulfate were established by paper chromatography in three solvent systems (15): n-butanol, water; n-butanol, morpholine, diethylene glycol, water; n-butanol, diethylene glycol, water. Each was in an ammonia atmosphere. Allantoin was isolated from the urine. The atom per cent excess C^{13} values, listed in Table II, are the average of duplicate determinations.

Degradation of Guanine Sulfate from Nucleic Acids—A 47.9 mg. sample of guanine sulfate was converted to guanidine picrate (6.3 mg.) by treat-

ment with 47.8 mg. of potassium chlorate in 3 ml. of concentrated hydrochloric acid according to the method previously used (2).

Toxicity of 2,6-Diaminopurine in Rats with Regenerating Liver—To determine possible toxic or inhibitory effects of 2,6-diaminopurine on rats with regenerating liver, two rats weighing 203 and 215 gm. were subjected to subtotal hepatectomy (13) and 6.15 and 4.03 gm. of liver were removed respectively. The animals had access to water and their usual Purina animal chow. 1 day after operation, the first rat was given by stomach tube 5 mg. (approximately 0.2 mM per kilo) of 2,6-diaminopurine dissolved in 1 ml. of water containing an equivalent quantity of lactic acid. The dose was repeated on the 2nd day and a double dose given on the 3rd day. The animal died several hours after the last administration. Autopsy revealed that liver regeneration was considerable and that the intestines appeared somewhat ischemic. Most of the intestine was devoid of any partially digested food or fecal matter. The second rat survived three equal doses of 5 mg. each. Liver regeneration was extensive, but the intestines showed a picture similar to that of the first rat.

Feeding of Isotopic 2,6-Diaminopurine to Rats with Regenerating Liver— Subtotal hepatectomies were performed on eleven adult male rats with an average weight of about 276 gm. (range 249 to 384 gm.) and an aggregate weight of 3038 gm. A total of 65.6 gm. of liver was removed and this amounted to about 78 per cent of the estimated total weight (84.1 gm.) of the whole livers (13). A 0.55 per cent suspension of 2,6-diaminopurine sulfate (5.45 atom per cent excess N^{15}) was prepared in 10 per cent gum acacia in water. Beginning on the day after operation, each rat was given via stomach tube 1 ml. of this suspension per day for 5 days. The daily dose was equivalent to 14.5 mg. of the free base per kilo of body weight per day. During the course of the experiment, the animals had free access to their usual food and water. The animals were killed with ether anesthesia on the 6th postoperative day. The total livers, weighing 92.1 gm., which were removed, indicated an average regeneration of 103 per cent. The intestines of most of the animals appeared ischemic and the small amount of food and fecal matter in the intestines was in suspension in relatively large amounts of fluid. However, the animals showed no diarrhea during the course of the feedings. The testes of most of the animals, which were all rather young adults, appeared rather small and atrophic.

The livers were homogenized in a Waring blendor in the cold with 920 ml. of ice-cold water and an equal volume of cold 14 per cent trichloroacetic acid was added. After half an hour, the insoluble portion was removed and washed with cold 1 per cent trichloroacetic acid, followed by water, acetone, and ether. The dried residue was defatted by boiling in a liter of ether. The residue was hydrolyzed with 800 ml. of 1 N KOH

at 37° for 20 hours and DNA and PNA fractions were prepared (13). The DNA was purified by a reprecipitation. The DNA and PNA fractions were hydrolyzed with 0.4 N HSO$_4$ and copper purines were prepared from the hydrolysates. The adenine and guanine obtained from the copper purines were characterized and found to be homogeneous by filter paper chromatography. The isotope values are listed in Table III.

DISCUSSION

In attempting to gain an insight into the mechanism of the known transformation of dietary adenine (I) to polynucleotide guanine (II), we have considered the conversion in terms of divers possibilities (Fig. 1). Since neither free hypoxanthine (V) nor xanthine (VI) functions as a precursor of nucleic acid purines in the rat (6), a preliminary hydrolysis of the 6-amino group of adenine (I) is thereby excluded. The occurrence of isoguanine (III) in nature (16, 17) encouraged the consideration of an oxidation of adenine (I) in the 2 position as the first step in its conversion

FIG. 4

to nucleic acid guanine (II). Since free isoguanine is not utilized by the rat for nucleic acid purine synthesis (5), this possibility is also excluded. Rather, the over-all conversion would appear to proceed from adenine (I) via an amination to yield 2,6-diaminopurine (IV). Although this postulate has the virtue of simplicity, it is unlikely that 2,6-diaminopurine (IV) would have resulted from a direct condensation of ammonia and adenine (I). Reichard (3) has found that the 2-amino group of guanine may be derived specifically from the nitrogen of glycine and thus a specific interaction between glycine and adenine might be involved.

The possibility that 2,6-diaminopurine represents an intermediate in a synthetic pathway which does not involve adenine cannot be excluded. However, the fact that the renewal of polynucleotide guanine is approximately the same after the feeding of either adenine or 2,6-diaminopurine discounts this possibility.

It must be emphasized that 2,6-diaminopurine does not lead to free guanine, since guanine itself is not incorporated into nucleic acids. At least two changes are necessary, and conjugation to ribose (or desoxyribose) might be the first step in the conversion of 2,6-diaminopurine into polynucleotide guanine (Fig. 4).

Various reports have pointed up the several potent biological effects

exhibited by 2,6-diaminopurine. It has been found to inhibit the multiplication of vaccinia virus *in vitro* (18), the estrogen-induced growth in the genital tract of the female chick (19), and the growth of *Lactobacillus casei* (20). Its administration may also lead to lesions of the intestinal mucosa and severe depletion of the hematopoietic elements in bone marrow (21). The effect on neoplasms deserves special mention. For example 2,6-diaminopurine inhibits the growth of sarcomas in tissue culture (22) and prolongs the life of mice with certain transplanted leucemias (23).

In addition to adenine, 2,6-diaminopurine represents the only other free purine known to participate in nucleic acid purine synthesis in the rat. Unlike adenine, diaminopurine gives rise exclusively to nucleic acid guanine. This fact should be taken into account whenever an explanation for the various physiological activities associated with 2,6-diaminopurine is to be considered. If the normal functioning and physiological integrity of the cell require the maintenance of a specific constitution for the various nucleic acids in the cell (for instance, a constant and characteristic guanine to adenine ratio), an explanation of the action of 2,6-diaminopurine might be suggested. This explanation would visualize that the structural integrity of the nucleic acids might be upset by a "flooding" of the tissues with the specific precursor of the one purine, and that there would be a concomitant disturbance in the normal functioning of the nucleic acids.

The alternative that diaminopurine may behave as an antimetabolite and inhibit the utilization of adenine receives considerable support from the studies with *L. casei* (20), chick oviduct growth (19), and in tissue culture (22), but the profound effects on intact animals (21, 23) have not been reversed by adenine.

A discussion of the question of whether 2,6-diaminopurine represents a "normal" intermediate or precursor in the biosynthesis of nucleic acid guanine is probably premature at this time. Appraisal must take cognizance of the pharmacological activity of the compound and of the fact that this purine has not, as yet, been shown to occur in nature.

SUMMARY
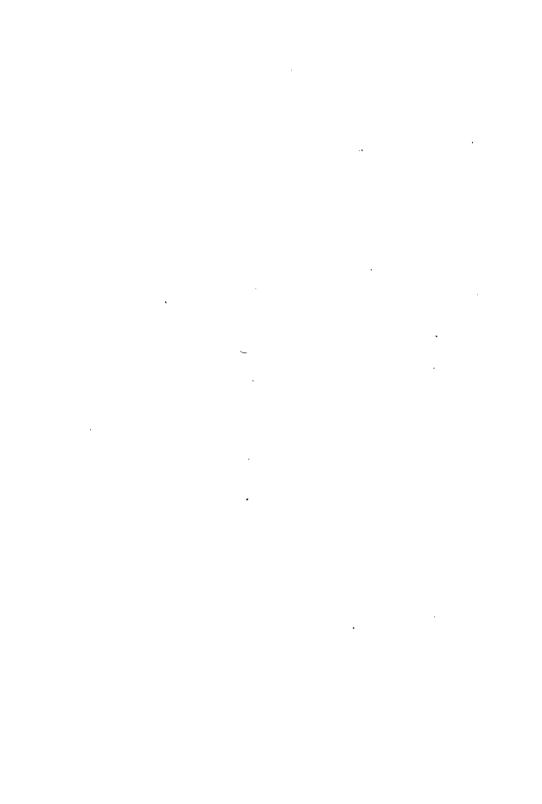
2,6-Diaminopurine has been synthesized with an excess of N^{15} in the 1- and 3-nitrogens as well as the 2-amino group. In another isotopic synthesis, excess C^{13} has been incorporated in the 2-carbon.

2,6-Diaminopurine is utilized by the rat for the biosynthesis of the guanine of both the pentose and desoxypentose nucleic acids, but does not lead to polynucleotide adenine.

The biochemical implications of this finding are considered in the light of the pharmacological actions of 2,6-diaminopurine.

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A STUDY OF THE METABOLISM OF
2,4-DIAMINOPYRIMIDINE*

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Recent studies have revealed that various derivatives of 2,4-diamino-pyrimidine exhibit profound biochemical reactivity. For example, nearly all 2,4-diaminopyrimidines and their condensed ring systems inhibit the growth of *Lactobacillus casei* with folic acid (1). 2,4-Diamino-5-*p*-chlorophenoxypyrimidine was found to be a potent antimalarial in that it was active against *Plasmodium gallinaceum* infection in chicks (2); the activity exhibited was of the same order as that of quinine. A definite chemotherapeutic effect of 2,6-diaminopurine[1] and 4-amino-N^{10}-methylfolic acid against transplanted mouse leucemia has been described (3, 4). Inhibition by 2,6-diaminopurine of the multiplication of vaccinia virus *in vitro* (5) and of estrogen-induced growth in the genital tract of the female chick has been observed (6).

In addition, this purine, 2,6-diaminopurine, was found to be utilized by the rat for nucleic acid guanine synthesis (7, 8).

It was therefore felt desirable to investigate the metabolic fate of the simplest member of this series and this communication will deal with a metabolic study of 2,4-diaminopyrimidine[1] and a report on its rôle in the biosynthesis of nucleic acids. The pyrimidines occurring naturally in nucleic acids, uracil (9), thymine (9), and cytosine (10), have been found to be inactive in this regard. On the other hand, orotic acid (4-carboxy-uracil) is an active precursor of nucleic acid pyrimidines (11).

When rats on an otherwise normal diet were permitted to ingest 2,4-diaminopyrimidine (12), labeled with an excess of N^{15} in the 1- and 3-nitrogen atoms as well as in the 2-amino group, the compound was extensively absorbed, as evidenced by the rather high N^{15} content of the urine (Table I). No evidence of incorporation into the tissue nucleic

[1] By the older numbering system, 2,4-diaminopyrimidine can be named 2,6-diaminopyrimidine. In any case, this compound and 2,6-diaminopurine have the same pyrimidine system in common.

acids was found. The compound was excreted undegraded, since the isotope levels of its possible urinary end-products, urea, ammonia, and allantoin, were identical with the isotope values of urea and allantoin from normal rat urine. This slight enrichment in isotopic nitrogen content of urinary constituents has been previously noticed in humans on normal diets (13).

TABLE I

Results from Isotopic 2,4-Diaminopyrimidine

Compound fed, 2,4-diaminopyrimidine, 8.03 atom per cent excess N^{15}.

Materials isolated	Atom per cent excess N^{15*}
Sodium nucleic acids...	0.006
Nucleic acids...	0.002
Purine hydrochlorides..	0.003
Silver pyrimidines...	0.004
Urea...	0.004
" control†..	0.005
Ammonia...	0.005
Allantoin	0.004
" control†..	0.004
Total urinary nitrogen...	0.186
" fecal nitrogen..	0.034

* Consolidated-Nier ratio mass spectrometer, model 21-201, probable error ±0.001; tank nitrogen used as standard.

† From the urine of rats on normal diets receiving no isotopic supplement.

EXPERIMENTAL

Preparation of Isotopic 2,4-Diaminopyrimidine (12)—Guanidine nitrate was prepared (14) from dicyandiamide and ammonium nitrate (containing about 32 atom per cent excess N^{15} in the ammonium radical).

Isotopic guanidine nitrate (2.44 gm.) and 3.0 ml. of cyanoacetaldehyde diethylacetal (10) were added to 18 ml. of absolute *n*-butanol in which 0.5 gm. of sodium had been dissolved, and the mixture was refluxed for 2½ hours with constant stirring. After the mixture was cooled, the precipitated $NaNO_3$ was removed and the filtrate was acidified with sufficient 6 N H_2SO_4 to give maximal precipitation of the crude pyrimidine, 2.94 gm.

The product, crystallized from 2 N H_2SO_4 and dried at 110° over P_2O_5 *in vacuo*, contained 8.03 atom per cent excess N^{15} (theory, 8 per cent).

$$C_4H_6N_4 \cdot \tfrac{1}{2}H_2SO_4 \cdot \tfrac{1}{2}H_2O. \quad \text{Calculated, N 33.3; found, N 32.9}$$

Three adult male rats of the Sherman strain with a combined weight of

900 gm. were fed, over a period of 3 days, a total of 306 mg. of non-isotopic 2,4-diaminopyrimidine sulfate admixed with 180 gm. of moistened stock diet (Rockland rat diet complete) to which the animals had been previously accustomed. Commencing on the 4th day, and for a week thereafter, the animals were maintained on a normal diet. During this period the daily excretions, both urinary and fecal, were collected and pooled. On the 11th day the animals were fed, over a period of 3 days, a total of 306 mg. of 2,4-diaminopyrimidine sulfate (8.03 atom per cent excess N^{15}) admixed with 180 gm. of stock diet. The daily urine and fecal excretions were collected. The animals were sacrificed on the day following the last isotope feeding. The urinary constituents and nucleic acids were isolated by the procedures outlined in previous studies (15). The feces, urinary constituents, and the total nucleic acids of the viscera were examined for isotope content. The normal urinary constituents were also examined as controls. The results are given in Table I.

The authors wish to acknowledge the analytical assistance of Mr. Roscoe C. Funk, Jr., and Mr. John Deonarine.

SUMMARY

A synthesis of 2,4-diaminopyrimidine, containing an excess of N^{15} in the 1 and 3 positions as well as in the 2-amino group, is described. Although the compound was extensively absorbed by the rat, the data presented indicate that 2,4-diaminopyrimidine is not metabolized to ammonia, urea, or allantoin, nor is it utilized for the biosynthesis of nucleic acids.

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A NEW SYNTHESIS OF THE PURINES ADENINE, HYPO-
XANTHINE, XANTHINE, AND ISOGUANINE

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The studies of Emil Fischer (1) on the chemical nature of the bases, adenine and guanine, derived from nucleic acids and of the alkaloids such as caffeine, theobromine, and theophylline obtained from plants showed them to possess a common bicyclic ring structure (I), consisting of a pyrimidine ring fused with an imidazole ring. The first total syntheses from simpler molecules were carried out by Traube (2) who formed first the pyrimidine ring, thereafter completing the imidazole portion of the structure. Subsequent syntheses have, in general, been modifications of Traube's method (3, 4). The alternative possibility of initially preparing the imidazole part of the purine structure has been appreciated by a number of workers who found, with few exceptions (5), that the necessary intermediates were difficult to obtain (6). Renewed interest in the synthesis of purines through imidazole precursors is provided by the implication of such precursors in the metabolic steps leading to purine biosynthesis (7). The recent synthesis of 4-amino-5-imidazolecarboxamide in good yield from cyanoacetic ester as described by Shaw and Woolley (8) overcomes such difficulties of inaccessible intermediates and has now been made the basis of a new total synthesis of purines.

Naturally occurring purines contain either a 6-oxy or 6-amino substituent as shown in generalized formulas II and III (where the 2-substituent, R, may be —H, —OH, —NH$_2$) and may therefore be thought of as derived from either 4-amino-5-imidazolecarboxamide or from 4-amino-5-imidazolecarboxamidine. The formation of purines was studied first with 4-amino-5-imidazolecarboxamide (IV) as the intermediate imidazole. When this base was heated in formamide at 185°, hypoxanthine (VI) was formed. Since the last step in the preparation of 4-amino-5-imidazolecarboxamide (8) was the cyclization of formamidomalonamamidine hydrochloride (VII) by heat, the possibility of converting the latter in a single operation to hypoxanthine in hot formamide recommended itself. This transformation was achieved in a yield of 62 per cent. The stepwise course of the reaction was indicated by the isolation, from a solution heated at 150° instead of 185°, of the intermediate 4-formamido-5-imidazolecarboxamide (V). The formamido compound was obtained more con-

veniently, however, from the aminoimidazole (IV) through the action of
formic acid and acetic anhydride. It is of interest to note that 4-form-
amido-5-imidazolecarboxamide (V) cyclizes readily to hypoxanthine (VI)
in aqueous solution in the presence of so weak an alkali as bicarbonate.
In acid solution, hydrolysis of the formyl group takes place and the free
aminoimidazole is liberated.

The synthesis of xanthine (IX) from 4-amino-5-imidazolecarboxamide
(IV) was readily accomplished in a yield of 75 per cent by fusion with
urea. This reaction had been carried out by Stetten and Fox (9) on the
base isolated from *Escherichia coli* before the chemical nature of the bac-
terial product was understood. In a conversion to xanthine in a stepwise
manner, the aminoimidazole was treated with ethyl chlorocarbonate and
the resultant 4-carbethoxyamido-5-imidazolecarboxamide (VIII) cyclized
to xanthine (IX) by means of heat or alkali.

A number of attempts were made to transform 4-amino-5-imidazole-
carboxamide (IV) to guanine by means of reagents commonly used to
convert amines to substituted guanidines, such as *S*-methylisothiourea,
cyanamide, and guanidine. As interpreted on the basis of paper chroma-
tography in an aqueous quinoline-collidine mixture (12) together with
ultraviolet spectroscopy, these reactions lead to the formation not only of

₃uanine, but also of xanthine and other unidentified insoluble products.
method suitable for the preparation of guanine was not found.

. For the preparation of 6-aminopurines, such as adenine, 4-amino-5-imidazolecarboxamidine (XIII) was desired. A synthesis of this compound was therefore undertaken by application of the novel ring closure used in the preparation of 4-amino-5-imidazolecarboxamide (8). Malonitrile was converted to the diamidine (X) which readily coupled with benzenediazonium chloride, producing phenylazomalonamidine (XI). In the reduction of the azo compound by means of zinc dust in formic acid, part of the formamidomalonamidine (XII) produced cyclized to the desired imidazole

(XIII). Rather than separate the products, the mixture of amidine hydrochlorides (XII and XIII) was thermally converted entirely to 4-amino-5-imidazolecarboxamidine dihydrochloride (XIII) at 170°. The yield was 25 per cent from malonitrile. The amidine has recently been reported as a hydrolytic degradation product of adenine (10).

As in the hypoxanthine synthesis, the aminoimidazole (XIII) was readily formylated in formic acid and acetic anhydride to 4-formamido-5-imidazolecarboxamidine (XIV). In hot bicarbonate solution, the pyrimidine ring closed, completing the synthesis of adenine (XV) in good yield. The tendency for the ring to close in the case of the amidine (XIV) was greater than for the corresponding amide. Recrystallization from water resulted in a considerable conversion to adenine. The cyclization also took place

in warm hydrochloric acid in contrast to the formamidoamide (V) which lost its formyl group under the same conditions.

When 4-amino-5-imidazolecarboxamidine dihydrochloride was fused with urea, isoguanine (XVI) was obtained.

The spectra in the ultraviolet region of the synthetic purines were measured and found to be in general agreement with published curves (11).

<div align="center">EXPERIMENTAL</div>

Melting points were determined in a copper block and are uncorrected.

Paper Chromatography—For an additional check on the identity of the synthetic purines and for a convenient means of studying the effect of reaction conditions, paper chromatography was of value. The method of Vischer and Chargaff (12) was employed except that upward migration of the solvent boundary was used. The compounds were applied to Whatman No. 1 filter paper along a line 3 cm. above the solvent level. The paper was formed into a cylinder and placed in a mixture of n-butanol (4 parts), diethylene glycol (1 part), and water (1 part). A beaker of N NH_4OH was present during the solvent rise which was allowed to proceed usually for about 16 hours.

Hypoxanthine—A solution of formamidomalonamamidine hydrochloride hemihydrate (8) (1.0 gm.) in formamide (15 ml.) was heated for $2\frac{1}{2}$ hours in a flask suspended in an oil bath at about 185°. The flask was then attached to a water pump and heating was continued until crystals began to separate. The cooled suspension was thinned with 95 per cent alcohol and filtered. The resultant dark powder was stirred with cold water (10 ml.) and the insoluble portion taken up in boiling water (60 ml.). The hot solution was filtered to remove colored impurities, concentrated to 25 ml., and allowed to stand at 4°. The resulting crystals were dried at 100° *in vacuo*, yielding 0.45 gm., 62 per cent. A solution of the product in phosphate buffer at pH 6.5 showed a single maximum absorption at 250 mμ, $\epsilon = 11,500$. On a paper chromatogram, a single spot, $R_F = 0.40$, was obtained, as with authentic hypoxanthine.

<div align="center">$C_5H_4ON_4$. Calculated, C 44.12, H 2.96; found, C 44.15, H 3.13</div>

When 4-amino-5-imidazolecarboxamide hydrochloride was treated with formamide at the same temperature for $1\frac{1}{2}$ hours, a 63 per cent yield of hypoxanthine was obtained.

4-Formamido-5-imidazolecarboxamide (V)—Formamidomalonamamidine hydrochloride hemihydrate (0.50 gm.) was heated in formamide (5 ml.) for 45 minutes in an oil bath at 150°. Most of the formamide was then distilled off *in vacuo*. The residual syrup was thinned gradually with 95 per cent alcohol until the separating crystals appeared to become gummy,

then filtered. The product, 0.25 gm., was recrystallized from water and dried at 100° *in vacuo*.

$C_5H_6O_2N_4$. Calculated, C 38.97, H 3.92; found, C 38.73, H 3.87

For preparative purposes, the following procedure was more suitable. 4-Amino-5-imidazolecarboxamide hydrochloride (1.63 gm.) and sodium formate (0.74 gm.) were dissolved in 98 per cent formic acid (5 ml.). Acetic anhydride (10 ml.) was added and an exothermic reaction took place, initiated by gentle warming. After 10 minutes, the mixture was heated in a water bath at 70° for an additional ½ hour, then taken to dryness *in vacuo*. The residue was stirred with water (15 ml.) to remove starting material and salts and filtered. The insoluble portion, 1.32 gm., 86 per cent, gave a single spot, $R_F = 0.50$, on the paper chromatogram and was therefore essentially free of hypoxanthine. Recrystallization of the material from water gave 0.87 gm.

When the formyl compound was heated for 15 minutes with methanolic HCl plus sufficient water to effect solution, 4-amino-5-imidazolecarboxamide hydrochloride was recovered from the solution, m.p. 255–256° with decomposition.

The formyl derivative showed a single maximum absorption in the ultraviolet region at 268 mμ, $\epsilon = 10,900$ at pH 6. In this respect it resembled the free amino compound. In addition, no neutral solvent mixture was found to distinguish between the base and its formyl derivative by paper chromatography. The formyl compound sublimed unchanged above approximately 220°. The imidazole compounds, unlike purines, are not precipitated by ammoniacal silver nitrate.

Cyclization of 4-Formamido-5-imidazolecarboxamide to Hypoxanthine under Alkaline Conditions—The formamido compound (0.75 gm.) was added to a solution of sodium (0.30 gm.) in absolute alcohol (50 ml.) and the suspension refluxed for 6 hours. Uncyclized formyl derivative remaining at this time was hydrolyzed by acidification with alcoholic HCl and continued refluxing for 10 minutes. After the mixture had been taken to dryness, the residue was dissolved in dilute KOH, treated with charcoal, and precipitated with acetic acid. The hypoxanthine thus obtained was recrystallized from water (25 ml.) to yield 0.43 gm., 65 per cent.

$C_5H_4ON_4$. Calculated, C 44.12, H 2.96; found, C 44.18, H 2.90

4-Formamido-5-imidazolecarboxamide was also readily cyclized to hypoxanthine in dilute aqueous alkali. The formyl compound (93 mg.) was refluxed in 0.05 N potassium bicarbonate (40 ml.) for 3½ hours. The solution was taken to dryness, and the residue stirred with a small volume of water. The insoluble portion was dissolved in NH₄OH and the solution

treated with 5 per cent silver nitrate. The precipitate was washed and decomposed with hydrogen sulfide in the usual manner. The resultant solution showed selective absorption at 249 mμ, and comparison with dilutions of known hypoxanthine concentrations indicated the formation of 70 mg. of hypoxanthine in the reaction, a conversion of 85 per cent. At 37°, ring closure of the formamido compound to hypoxanthine can also be observed in bicarbonate solution by the above method.

Xanthine—4-Amino-5-imidazolecarboxamide hydrochloride (0.30 gm.) and urea (0.30 gm.) were fused together for 2 hours in an oil bath at 175°. The cooled melt was ground up with added water. The crude xanthine was filtered and washed. A solution of the product in N KOH was decolorized with charcoal, and the xanthine precipitated by the addition of acetic acid. Finally, the product was recrystallized by concentration of a strongly ammoniacal solution to half volume, yielding 0.21 gm., 75 per cent. The sample was dried *in vacuo* at 125° for 2 hours.

$C_5H_4O_2N_4$. Calculated, C 39.48, H 2.65; found, C 39.38, H 2.81

A solution of the product in phosphate buffer at pH 6.5 had maximum absorption at 268 mμ, ϵ = 10,800. Like an authentic sample of xanthine, this material gave an R_F value of 0.28 on the paper chromatogram.

4 - Carbethoxyamido - 5 - imidazolecarboxamide—4 - Amino - 5 - imidazolecarboxamide hydrochloride (0.82 gm.) was stirred at 0° with a solution of potassium bicarbonate (0.60 gm.) in water (15 ml.). At 10 minute intervals, three 0.2 ml. portions of ethyl chlorocarbonate were added with additional potassium bicarbonate (0.2 gm. per 2 ml.). Stirring was continued for 1 hour after the last addition. The separated crystals were filtered and combined with an additional small crop obtained on treating the filtrate with acetic acid, yielding 0.36 gm., 36 per cent. The product was recrystallized from water.

$C_7H_{10}O_3N_4$. Calculated.   C 42.42, H 5.08, N 28.27
              Found.   " 42.27, " 5.21, " 28.54

The product melted at about 175°, with foaming and resolidification, due to the formation of xanthine. Analytically pure xanthine was obtained when the carbethoxyamido compound was refluxed in nitrobenzene for 10 minutes, followed by recrystallization. The same change was brought about by heating in concentrated NH$_4$OH at 100°. The identity of the products was checked by paper chromatography and spectroscopy.

Phenylazomalonamidine Dihydrochloride—Malonitrile and 2 molar equivalents of absolute ethanol in dioxane solution were saturated at 0° with hydrogen chloride as described by McElvain and Schroeder (13). The resultant imino diethyl ether dihydrochloride on treatment with alcoholic

ammonia gave malonamidine dihydrochloride (14) in a yield of 60 per cent for the two steps.

Aniline (9.6 ml.) dissolved in 6 N hydrochloric acid (60 ml.) was diazotized below 5° by slow addition of a solution of sodium nitrite (8 gm.) in water (20 ml.). 15 minutes after the addition had been completed, the solution was mixed with malonamidine dihydrochloride (17.3 gm.) in water (75 ml.). The pH was adjusted to about 4 by the slow addition of powdered potassium bicarbonate (20 gm.) followed by sufficient concentrated sodium acetate. The reaction mixture was left overnight at room temperature, subsequently freed of some dark gum by filtration, and concentrated *in vacuo* until thickened by crystallization. The fine, yellow needles of azo compound were filtered with suction and washed twice with 6 N HCl. A second crop of azo compound may be obtained by the slow addition of concentrated HCl to the filtrate. Such material is contaminated with inorganic chlorides and should be recrystallized from a small volume of water. The product is quite soluble in water. Dried to constant weight *in vacuo* over NaOH, the yield of azo compound is very nearly quantitative. For analysis, a sample was recrystallized several times from water and dried for 2 hours *in vacuo* at 78°. The results indicate hydration.

$C_9H_{12}N_6 \cdot 2HCl \cdot \frac{1}{2}H_2O$.   Calculated.   C 37.77, H 5.28, Cl⁻ 24.8
Found.   " 37.65, " 5.35, " 24.4

4-Amino-5-imidazolecarboxamidine   *Dihydrochloride*—Phenylazomalonamidine dihydrochloride (10 gm.) in 98 per cent formic acid (100 ml.) was treated gradually with zinc dust (10 gm.) at 40–45°. The colorless reaction mixture was filtered. The filtrate, combined with formic acid washings of the insoluble material, was concentrated to a syrup *in vacuo*. After the addition of water, the concentration was repeated to complete the removal of formic acid. An aqueous solution of the residue was freed of zinc by means of hydrogen sulfide, and the filtrate taken once more to a syrup *in vacuo*. The crude reaction product was now precipitated by dissolving the syrup in methanol (25 ml.) and adding anhydrous ether (200 ml.). The crude dihydrochloride, dried over sodium hydroxide in a vacuum desiccator, was heated for 15 minutes in an oil bath at 170–180°. The melt was dissolved in a minimum amount of water and treated with absolute alcohol and ether. The initial precipitates contained most of the pigmented impurities and were discarded. Continued precipitation yielded, finally, 4.2 gm. of crystals, m.p. 238–242°. Recrystallization gave 3.3 gm., m.p. 242–244°, 45 per cent.

$C_4H_9N_5Cl_2$.   Calculated.   C 24.25, H 4.58, Cl⁻ 35.8
Found.   " 24.08, " 4.46, " 36.3

If the crude hydrochloride from the zinc dust reduction were subjected to

crystallization, instead of thermal conversion to the imidazole as described, crystals could be obtained which appeared to consist of formamidomalonamidine (XII) mixed with imidazole (XIII). Thus, when the gummy product was triturated with a small volume of methanol, crystallization slowly proceeded and was completed by the slow addition of ether, yielding 4.3 gm., m.p. about 240°. However, if the sample was inserted into a preheated block at 150°, foaming at about 165° was observed with re-solidification of the melt and eventual final melting at 240°. The pure imidazole amidine undergoes no preliminary change at 165° observed in the same manner. When taken in the usual way, the melting point observed for formamidomalonamidine is that of the imidazole formed during the heating. That some imidazole was present at the start was indicated by some selective absorption at 285 mμ.

4-Amino-5-imidazolecarboxamidine dihydrochloride obtained in the above synthesis gave a single maximum absorption at 285 mμ, $\epsilon = 11,300$, pH 6.5.

Adenine—4-Amino-5-imidazolecarboxamidine dihydrochloride (0.99 gm.) and sodium formate (0.70 gm.) were dissolved in 98 per cent formic acid (10 ml.) and treated with acetic anhydride (10 ml.). After a lag of 10 minutes, an exothermic reaction occurred, bringing the mixture to the boiling point. When this had subsided, the solution was warmed at 70° for an additional 15 minutes, then taken to a syrup *in vacuo*. The residue crystallized when stirred with water and was filtered, yielding 0.70 gm. of 4-formamido-5-imidazolecarboxamidine. The base showed a single maximum in the ultraviolet at 272 mμ; a slight contamination with adenine was suggested by the shape of the curve.

4-Formamido-5-imidazolecarboxamidine (0.20 gm.) was refluxed for 1 hour in 0.5 N potassium bicarbonate (10 ml.). The solution was neutralized, concentrated to 2 ml., and left at 4° to crystallize. The filtered crystals, washed with water and dried in air at 100°, weighed 0.155 gm., a yield of 80 per cent for the two steps from 4-amino-5-imidazolecarboxamidine dihydrochloride. For analysis, a sample recrystallized from water was dried to constant weight *in vacuo* at 125°.

$C_5H_5N_5$. Calculated, C 44.45, H 3.73; found, C 44.26, H 3.82

The base formed a picrate, m.p. 286–287°, undepressed by admixture with the picrate of an authentic sample. On a paper chromatogram, both synthetic and natural bases gave $R_F = 0.58$. In phosphate buffer at pH 6.5, the synthetic adenine showed a single maximum absorption in the ultraviolet at 261 mμ, $\epsilon = 13,600$.

Isoguanine—4-Amino-5-imidazolecarboxamidine dihydrochloride (0.30 gm.) and urea (0.30 gm.) were mixed and heated for 2 hours in an oil bath

at 160°. The initial melt gradually solidified. After cooling, the mixture was thoroughly triturated with water, washed, and dried. The product, 0.22 gm., was recrystallized by concentrating a solution of the base in strong ammonium hydroxide at the boiling point until crystals began to form. The material which separated from the chilled solution was washed and dried *in vacuo* at 125° for 3 hours.

$C_5H_6ON_5$. Calculated, C 39.75, H 3.34; found, C 39.67, H 3.30

In phosphate buffer at pH 6.5, the synthetic isoguanine exhibited two maxima in the ultraviolet: at 240 mμ, ϵ = 8000, and at 286 mμ, ϵ = 9850.

SUMMARY

The preparation of imidazole intermediates and the conversion of these to purine bases have been carried out easily and with high yields by new methods which offer a practical route of purine synthesis. The synthesis of 4-amino-5-imidazolecarboxamidine in an over-all yield of 25 per cent from malonitrile has been accomplished. This imidazole, and the corresponding carboxamide, have each been condensed with formic acid and the resulting 4-formamido-5-imidazolecarboxamidine and carboxamide cyclized to adenine and hypoxanthine, respectively. When the amino-imidazoles were heated with urea, the amidine lead to isoguanine; the amide, to xanthine.

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THE METABOLISM OF ACETONE

I. GROSS ASPECTS OF CATABOLISM AND EXCRETION*

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Early evidence for the metabolic activity of acetone was derived from studies showing a partial disappearance of administered doses of the compound (1, 2). It is clear from these investigations, and from subsequent work (3, 4), that most of the acetone excreted as volatile iodoform-forming substance appears in the exhaled air; a smaller fraction is excreted in the urine. Schwarz (1) demonstrated that the fraction of administered acetone recoverable in the breath becomes smaller as the size of the administered dose is decreased. About 55 per cent of the acetone was exhaled and about 1.2 per cent was excreted in the urine when doses of 300 to 600 mg. per kilo were administered to healthy dogs; only 18 per cent was exhaled when the dose was 3.5 mg. per kilo. These findings are compatible with the view that any acetone, if formed in small amounts in normal metabolism (1, 4–7), is largely metabolized.

Apparent conversions of acetone to acetoacetic acid (7), β-hydroxybutyric acid and lactic acid (8), and glucose (9) have been reported. However, Koehler *et al.* (7) found no increase in the blood concentrations of β-hydroxybutyric acid or glucose when human subjects were given 10 gm. of acetone intravenously. Recently Borek and Rittenberg (10) have demonstrated the incorporation of deuterium from deuterioacetone into the cholesterol of rat liver slices. This observation provided conclusive evidence of metabolic activity of acetone in this tissue.

Deuterioacetone is unsatisfactory for quantitative studies of acetone metabolism because an unknown fraction of the deuterium may be lost by enolization of the acetone and its potential metabolic products. $C^{14}H_3$-$COC^{14}H_3$ was used in the present studies.

Synthesis of Radioacetone

Methyl-labeled acetic acid was prepared from C^{14}-methanol[1] by the Grignard reaction with carbon dioxide.[2] An aliquot of the acetic acid was converted to silver acetate and decarboxylated (11); the carbon dioxide formed was inactive.

5.24 mM (equivalent to about 0.3 mc.) of the labeled acetic acid were titrated with standard barium hydroxide to a phenolphthalein end-point. A few drops excess of barium hydroxide were added and the solution evaporated to dryness *in vacuo*. By vacuum pyrolysis at 500° the barium acetate was converted to acetone which was condensed in a refrigerated trap and precipitated as the 2,4-dinitrophenylhydrazone by the method of Iddles and Jackson (12); m.p. 122–123°. The product was hydrolyzed with 6 N sulfuric acid and the acetone distilled off together with 19 gm. of water. This aqueous solution,[3] suitably diluted, was employed in the biological experiments.

A portion of the acetone was precipitated with 2,4-dinitrophenylhydrazine and the weight of dinitrophenylhydrazone, corrected for a 4 per cent loss by solubility, was used for calculating the acetone concentration in the aqueous solution and the yield of acetone from acetic acid (80 per cent). The melting point of the radioactive dinitrophenylhydrazone was 124.5–125.0°, identical with that obtained from distilled non-isotopic acetone.

Analysis—$C_9H_{10}O_4N_4$ (238.2). Calculated. C 45.4, H 4.2
Found. " 46.0, " 4.3

The specific activity of the acetone was 7.3×10^5 c.p.m. per mg. A sample was suitably diluted with ordinary acetone and degraded to iodoform; the corrected specific activity of iodoform from the radioacetone was 1.87×10^6 c.p.m. per mg. of carbon (calculated 1.77×10^6). This shows that the acetone activity resided in the methyl groups of the acetone.

Methods

Collection of Exhaled Gases—In all experiments the rat was given an aqueous acetone solution by metal stomach tube 1 hour after deprivation of food and water. To obtain the exhaled radioactive acetone and carbon dioxide the rat was placed in a desiccator with an air inlet preceded by soda lime and aqueous barium hydroxide traps and an aqueous calcium

[1] The C^{14}-methanol was obtained from the United States Atomic Energy Commission.

[2] We wish to thank Dr. M. Engelman for the labeled acetic acid.

[3] Appreciable volatilization of the acetone occurs from such solutions when stored in a weighing bottle in the refrigerator. Standard acetone solutions should be kept in sealed glass tubes.

chloride dehumidifier, and followed by an air outlet in series with two ice-cooled acetone traps containing 2,4-dinitrophenylhydrazine solution (600 mg. in 200 ml. of 2 N HCl), three CO_2 traps containing nearly saturated barium hydroxide solution, a T-tube with a needle valve air bleeder, and a water aspirator. Air was drawn through the system at a rate of one to four bubbles per second.

Acetone in Exhaled Air—Since the amount of acetone exhaled by healthy rats during an experimental interval is not sufficient to provide a suitable deposit of dinitrophenylhydrazone, a measured amount of carrier acetone, enough to provide a theoretical yield of about 250 mg. of precipitate, was added to the cold acetone traps just before they were inserted into the train. Since the total amount of dinitrophenylhydrazone produced by the exhaled acetone was small, and the quantity of acetone lost by aeration was also found to be negligible, the total weight of derivative was calculated from the amount of added inactive acetone, the output of C^{14} in a sample being calculated from activity measurements on a convenient fraction of the total precipitate, usually an "infinitely thick" layer.

Carbon Dioxide in Exhaled Air—The samples from the three baryta traps were generally combined, filtered rapidly on a Büchner funnel, washed with distilled water, dried at 160° (13), weighed, and ground in a mortar, and portions placed in copper dishes for counting. A correction factor[4] of 0.834 was applied to correct for back-scattering.

Counting Methods—Sample and background activities were determined with Geiger-Müller end window counters, counts being measured until the net standard deviation values did not exceed ±5 per cent. Exceptions are Experiment A, in which some deviations are as great as ±20 per cent, and all values of which the specific activity is reported as less than 2 c.p.m. per mg. Whenever feasible, samples were prepared as layers of at least 50 mg. per sq. cm. in copper dishes of 2.06 cm. diameter. However, small samples (as low as 5 mg.) could be deposited in a reproducible manner only by using a modification[5] of the Henriques filtration technique (14) whereby a deposit of either 0.8 or 1.6 cm. diameter was formed on filter paper. Counting rates registered by deposits on paper were corrected to the standard area by application of conversion factors obtained from count-

[4] The factor for "infinitely thick" layers was calculated from activities found after small weights of $BaC^{14}O_3$ were ground to constant specific activities with (a) large amounts of glycine and (b) large amounts of barium carbonate. This factor is in agreement with the factor 0.83 obtained by N. Radin in this laboratory. His value was obtained by combustion of C^{14}-glycine to C^{14}-barium carbonate.

[5] The modification involves use of a rubber adapter made from a baby bottle nipple to support the glass cylinder and provide a vacuum-tight seal. To prevent curling of the filter papers while drying, they were covered with a weight placed on the margin of the paper.

ing rates registered by 0.8, 1.6, and 2.06 cm. deposits of C^{14}-barium carbonate. The very small deposit size was used only for some of the acetaminophenylbutyric acid samples. Measured count values were corrected for self-absorption by the method of Yankwich et al. (15). Specific activity values subsequently reported in this paper are expressed as counts per minute *per mg. of carbon.*[6]

Isolation Procedures—Acetyl-L-α-amino-γ-phenylbutyric acid was isolated from urine as previously described (16) and the samples were recrystallized from water (one to three times) until the melting point was 179–180°. The specific activity did not change significantly on recrystallization. In the final crystallization the solution was cooled quickly in an ice bath to obtain small crystals suitable for preparation of a uniform deposit on a radiochemical filter. These samples and the silver acetate obtained from them by acid hydrolysis gave the same calculated specific activities for the acetyl carbon.

Fatty acids were isolated by the usual methods. Liver cholesterol was isolated and counted as the digitonide; carcass cholesterol (m.p. 147°) was also isolated via this derivative.

Glycogen was isolated from rat liver by the procedure of Ostern and Hubl (17).

Hemin was isolated from 4 ml. of blood, by the method of Nencki and Zaleski (18).

The amino acids in Experiment B were isolated from the decapitated, skinned, eviscerated, and defatted carcass by the usual procedures (19).

EXPERIMENTAL

Experiment A—A 189 gm. female Wistar rat was given 0.22 mg. of acetone, equivalent to 7.5×10^4 c.p.m. in 1 ml. of water. Food[7] was made available *ad libitum* immediately after the dose had been administered. Respiratory CO_2 was collected for 13.5 hours. The results of this experiment are shown in Table I.

Experiment B—A 152 gm. female Wistar rat was given 1.08 mg. of radioacetone equivalent to 7.9×10^5 c.p.m. at 0.0 hour and at 24 hour intervals thereafter. Immediately after the first acetone administration the animal commenced to ingest food containing 1 per cent of added DL-α-amino-γ-phenylbutyric acid. This diet was maintained throughout the experiment. At the end of 7.67 days the rat was anesthetized with ether and sacrificed by cutting the thoracic aorta. About 4 ml. of blood were withdrawn from the thoracic cavity. The liver was quickly excised for the separation of glycogen; this separation was accomplished within 10

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[6] Specific activity values can be conveniently converted to "counts per minute per standard 2 cm. dish of carbon" by multiplying the reported values by 10.

[7] Rockland rat diet was used in all experiments.

minutes after the animal was sacrificed. The internal organs, skin, and head were then removed and the remaining carcass was shredded in a Waring blendor in the presence of 95 per cent ethanol.

The data of Experiment B are presented in Tables II and V.

The output of activity as carbon dioxide was 67 to 76 per cent; over 60 per cent was exhaled within 12 hours after the dose was given. The average specific activity for the first 7 days was 216 c.p.m. per mg. Acetate isolated from a composite urine sample representing 20 per cent of each daily excretion had a specific activity of 360 c.p.m. per mg.

TABLE I

Experiment A; Acetone Oxidation by Normal Rat

0.22 mg. of radioacetone* administered to a 189 gm. rat.

Time	Carbon dioxide exhaled	
hrs.	*c.p.m. per mg.*	*per cent of administered activity*
0.0– 0.5	43	2.8
0.5– 1.5	110	13
1.5– 2.5	100	13
2.5– 3.5	74	7.8
3.5– 4.5	30	2.4
4.5– 6.5	23	4.8
6.5–13.5	4	3.6
Total...		47.4

* In this experiment the specific activity of the administered acetone was 3.4×10^5 c.p.m. per mg. (or 5.5×10^5 c.p.m. per mg. of carbon) due to inferior counter geometry.

Experiment C—A 182 gm. male Sprague-Dawley rat, which had been rendered diabetic by injection into the caudal vein of 40 mg. of alloxan 35 days before the experiment began,[8] was kept on a stock diet without insulin therapy. At the time of the experiment the rat was passing 80 ml. of urine daily; the glucose concentration was 7 per cent.

During the period 6 to 2 hours before acetone administration the rat ingested stock diet containing 48 mg. of aminophenylbutyric acid. In addition, a water slurry equivalent to about 50 mg. of this foreign amino acid was administered by stomach tube 1 hour before acetone administration. At 0 hour 1.12 mg. of radioacetone, equivalent to 8.2×10^5 c.p.m., were administered. The acetone and carbon dioxide were collected as before. The data from this experiment are presented in Table III.

Experiment D—A 154 gm. female Wistar rat was given aminophenyl-

butyric acid and 1.12 mg. of radioacetone on the same schedule as that employed in Experiment C. Acetaminophenylbutyric acid was isolated from urine samples taken at 12, 24, and 40 hours. The specific activity values of acetate carbon were 703, 25, and 0 c.p.m. per mg. respectively.

Experiment E—After a 1 hour abstinence from food and water, a 210 gm. male rat was given 1.3 mg. of radioacetone equivalent to 9.5×10^6

TABLE II

Experiment B; Exhalation of Acetone and Its Conversion to Carbon Dioxide and Acetylating Agent

1.08 mg. of radioacetone administered to a 152 gm. rat at 24 hour intervals.

Sampling time	Acetone	Carbon dioxide		Acetate*
days	*per cent of daily administered activity*	*c.p.m. per mg.*	*per cent of daily administered activity*	*c.p.m. per mg.*
0.00 –0.014	0.67			
0.014–0.25	5.8	620	56	
0.25 –0.50	0.43	62	5.0	
0.50 –0.75		43	3.3	
0.75 –1.0		42	3.3	270
1.0 –1.5		350	67	
1.5 –2.0		31	4.7	360
2.0 –2.5		350	67	
2.5 –3.0		21	3.1	300
3.0 –3.5	3.76	380	68	
3.5 –4.0	0.17	45	6.4	
4.0 –4.5	4.58	350	69	
4.5 –5.0		30	4.7	
5.0 –6.0		220	76	320
6.0 –7.0		210	74	310
7.0 –7.62		242†	67	630
7.62 –7.66		29	0.5	

* Collected on a daily basis; see the text.

† The 7.62 day carbon dioxide sample was contaminated with atmospheric CO while present as $BaCO_3$ in $Ba(OH)_2$; hence the specific activity is low.

c.p.m. The 2,4-dinitrophenylhydrazone samples were purified by hydrolysis and distillation from 6 N H_2SO_4 and reprecipitation. Results from this experiment are shown in Table IV.

DISCUSSION

In agreement with the reports of earlier workers (1, 4), our studies show that very little acetone is excreted through the lungs when small doses of the compound are administered. Rats which had received radioacetone at a level of less than 1 mg. per 100 gm. exhaled a maximum of 7 per cent

TABLE III

Experiment C; Exhalation and Oxidation of Acetone by an Alloxan-Diabetic Rat

1.12 mg. of radioacetone administered to a 182 gm. rat.

Sampling time	Acetone	Carbon dioxide	
hrs.	*per cent of dose*	*c.p.m. per mg.*	*per cent of dose*
0.00– 0.33	0.32	193	0.43
0.33– 0.67	2.38	591	6.01
0.67– 1.00	1.42	748	4.84
1.00– 1.33	0.62	767	2.92
1.33– 1.67	0.73	817	4.48
1.67– 2.00	0.90	812	8.13
2.00– 2.33	0.34	615	4.38
2.33– 2.67	0.34	505	4.98
2.67– 3.00	0.07	338	3.00
3.00– 3.33	0.11	226	2.52
3.33–11.3	0.06	38	6.61
11.3 –19.3	0.00	17	2.67
19.3 –24.0	0.00	14	0.81
Total.............................	7.29		51.78

Acetate was isolated as acetaminophenylbutyric acid from pooled urine samples collected during the periods 0 to 12, 12 to 24, and 24 to 40 hours. The specific activity values for the acetate carbon of these samples were 414, 8, and 0 c.p.m. per mg. respectively.

TABLE IV

Experiment E; Acetone Exhalation by Normal Rat

1.30 mg. of radioacetone administered to a 210 gm. rat.

Sampling time	Acetone	Integrated acetone activity
hrs.	*per cent of dose*	*per cent of dose*
0.33	0.045	0.045
0.67	0.144	0.189
1.00	0.106	0.295
1.50	0.683	0.978
2.00	2.03	3.008
2.50	0.640	3.648
3.00	0.320	3.968
4.00	0.188	4.156
5.50	0.060	4.216
0.00–5.5*	0.024	4.24

* Composite sample in the second trap which was not changed.

of the radioactivity as acetone (Tables II and IV). The exhalation attained a maximum rate at about 2 hours after the dose was given and

decreased exponentially to a low value by the 6th hour.   It is clear from these data that, if small amounts of acetone are formed in normal metabolism, volatilization into the respiratory air is not an important disposal mechanism.   The slow disappearance of the radioacetone from the blood, as indicated by its prolonged presence in the respiratory gases, when related with its negligible concentration in normal blood (5, 20), suggests that little acetone is formed in normal metabolism.   When large amounts are formed or administered, an increase in blood acetone concentration occurs.   'The suggestion by Koehler, Windsor, and Hill (7), that the well established, slow disposal of *large* doses of acetone may be caused by derangement of acetone metabolism incident to the presence of the administered dose, cannot reasonably be applied to the present work in which doses of less than 1 mg. per 100 gm. were used.   Even minute quantities of acetone are here shown to be removed slowly, compared, for example, to pyruvate and lactate.

The observation that administered acetone cannot be entirely recovered in the respiratory gases and urine, and that only a trace is excreted in the feces (1) and sweat (2), led several workers to suggest that some of the acetone must be metabolized.   Conclusive proof of this interpretation is presented in the data of Tables I, II, and III, which show that a major fraction of the radioactive acetone administered is oxidized to carbon dioxide.   In Experiment B (Table II) wherein radioacetone was administered at 24 hour intervals, an average of 72 per cent of the methyl carbon of the administered acetone was daily oxidized to carbon dioxide.   The radioactive carbon dioxide reaches a maximum concentration in the exhaled air about 2 hours after the dose is given and drops to a low, slowly diminishing quantity after the 12th hour (Table II).

The preceding paragraphs indicate that, if acetone is formed in normal rats, such formation is quantitatively small, and the acetone formed must be metabolized slowly but with considerable efficiency.

Extensive utilization of acetone carbon atoms for acetylation is shown by the data of Experiments B and D.   After the ingestion of radioacetone equal to 8×10^5 c.p.m., the specific activity of the acetate carbon in the isolated acetaminophenylbutyric acid was about 350 c.p.m. per mg. as calculated on a daily basis.   Acetate is a possible source of this very active acetyl group.   Studies on isotope dilution of labeled acetate in rats indicate that the daily turnover of acetate in the rat is about 1 gm. per 100 gm. of body weight (21).   The labeled acetate from acetone in the 150 gm. rats used would thus be merged with a metabolic pool equivalent to 600 mg. of acetate carbon.   The total activity of this pool would then be $600 \times 350 = 2.1 \times 10^5$ c.p.m.   This activity corresponds to 27 per cent of that in the administered radioacetone, implying conversion of approximately this percentage of the acetone to acetate.   The results of Experiment D show

that the acetylating agent formed from acetone is rapidly metabolized. It is probable that the slight activity in the 12 to 24 hour sample is due to incomplete voiding during the first 12 hours.

The data of Experiment C (Table III) show conclusively that a diabetic animal can also oxidize acetone. There appears to be a marked shortening of time required for attainment of a maximum rate of acetone exhalation in the diabetic rat, most radioacetone being exhaled in the 20 to 40 minute sample instead of in the 100 to 120 minute sample as observed in normal rats. Oxidation of acetone to carbon dioxide is only about 70 per cent as high as in the normal rat of Experiment B (Table II).

TABLE V

Experiment B; Biosynthesis of Metabolites from Methyl Carbons of Acetone

1.08 mg. of radioacetone administered to a 152 gm. rat at 24 hour intervals.

Compound	Specific activity of total C	Compound	Specific activity of total C
	c.p.m. per mg.		*c.p.m. per mg.*
Urea in urine, composite for 7.6 days	250	Liver glycogen	40
		Aspartic acid	38
Liver cholesterol	123	Arginine	12
Carcass cholesterol	63	Carcass fatty acids	10
Glutamic acid*	56	Leucine fraction	2
Heme	48	Tyrosine	1

* All the amino acids listed were isolated from the eviscerated carcass.

Approximately 20 per cent of the administered activity was not accounted for by the exhaled carbon dioxide and carbonyl compounds in Experiment B. That a considerable percentage of the methyl carbon atoms of acetone enters into many metabolic reactions including sterol synthesis and syntheses generally associated with the tricarboxylic cycle is indicated by further data from Experiment B presented in Table V.

The ratio of specific activities, carcass cholesterol-carcass fatty acids, is about 6:1. Ratios of 0.4:1 to 1.7:1 have been reported in earlier studies with mice (22) and rats (23) with use of carbon-labeled acetate. The high ratio suggests that acetate from the oxidation of acetone may not have been the sole source of radioactivity found in the cholesterol. It is possible that some of the carbon atoms of cholesterol may be derived from acetone by another route. Acetone itself or some oxidation product such as methylglyoxal may condense with a C_2 unit to produce an intermediate (24) capable of further condensation to a sterol. The oxidation of acetone to acetate may give rise to a C_1 compound which might be directly employed. These mechanisms are being investigated.

The activities of the glutamic and aspartic acids[9] (Table V) may be presumed to have originated by condensation of labeled acetate with oxalacetate and equilibration of the subsequently formed tricarboxylic cycle intermediates with the metabolic pool of these amino acids. Our findings are in accord with this view, since the glutamic acid is somewhat more active than the aspartic acid.

The activities of the heme[10] and glycogen cannot be fully explained on the basis of present published data. Acetate is regarded as at least a partial source of the C^{14} label found in both compounds. The activity of the urea carbon from the pooled urine, as anticipated from the findings of Mackenzie and du Vigneaud (25), corresponds closely to the average CO_2 carbon activity for 7.67 days. Arginine activity can arise partially from incorporation of active bicarbonate in the amidine group and probably also by incorporation, via ornithine, of active glutamic acid carbon atoms in some of the other positions. The leucine fraction and tyrosine possess negligible activities.

SUMMARY

1. Small doses (0.1 to 0.6 mg. per 100 gm. of body weight) of acetone labeled with C^{14} in the methyl positions were administered to rats by stomach tube.

2. At the dosage level employed, radioactive carbonyl compounds excreted through the lungs comprised less than 10 per cent of the administered radioactivity. At least half of the activity was oxidized to carbon dioxide and exhaled in the respiratory air within 24 hours. The results show that although acetone is extensively oxidized in the normal intact rat the oxidation is slow compared to that of metabolites on major metabolic paths.

[9] Degradation studies made on these radioactive amino acids suggest that the Schmidt reaction, as described by Adamson (26) for glutamic acid, may be used as a one-step degradation method for specific removal of the γ-carboxyl group of glutamic acid. Barium carbonate obtained by the Schmidt reaction was only 7 per cent as active as barium carbonate from the α-carboxyl group, thus placing an upper limit of 7 per cent on the fraction of α-carboxyl CO_2 released by hydrazoic acid. It is probable that no significant α- or β-decarboxylation occurs when the Schmidt reaction is applied to α-amino acids, for no carbon dioxide was evolved when we applied the Adamson technique to aspartic acid.

[10] Radin et al. (27) incubated some of the radioacetone with duck blood and found no activity in the isolated hemin. That biosynthesis of heme occurred was demonstrated by the presence of N^{15}, from labeled glycine, in the hemin. We separated CO_2 from this blood after the incubation and found it to be inactive, indicating that the medium cannot completely oxidize acetone. A large amount of radioacetone remained after the incubation but it was not evaluated quantitatively. These findings suggest that an oxidation product of acetone, rather than acetone *per se*, is the heme-forming intermediate in the rat.

3. An alloxan-diabetic rat was also found capable of oxidizing acetone.

4. Extensive conversion of acetone to acetylating agent was shown.

5. Acetone carbon atoms were found to enter the following compounds arranged in order of decreasing carbon specific activity: urea, liver cholesterol, carcass cholesterol, glutamic acid, heme, liver glycogen, aspartic acid, arginine, and carcass fatty acids. The significance of these conversions is discussed.

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ACETOACETIC ACID FORMATION IN VITRO FROM ODD AND EVEN NUMBERED RADIOACTIVE FATTY ACIDS*

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Incubation of carboxyl-labeled fatty acids ($RC^{14}OOH$) with liver slices gave rise to $C^{14}O_2$ and labeled acetoacetic acid (1, 2). In the case of even numbered acids, much more of the radiocarbon appeared in the keto acid than was oxidized to $C^{14}O_2$; however, the reverse situation occurred when acids of an odd number of carbons were used (2). Previously, studies made with non-labeled acids had yielded similar differences between Q_{O_2} and total acetoacetic acid production (3). The acetoacetic acid formed from carboxyl-labeled octanoic acid (1, 4, 5) and hexanoic acid (4) has been shown to be asymmetrically labeled with respect to the carboxyl and carbonyl groups. That the acetoacetic acid formed by washed liver homogenates was the result of recondensation of 2-carbon fragments has been demonstrated (5, 6). In the latter reports, a mechanism was suggested to explain the asymmetry of the acetoacetic acid.

In the present paper further studies on the metabolism of radioactive fatty acids by liver and kidney slices are presented in which the radiocarbon respired in the CO_2 and present in the carbonyl and carboxyl groups of acetoacetic acid was determined. The substrates used were carboxyl-labeled pentanoic, hexanoic, heptanoic, octanoic, nonanoic, and dodecanoic acids, and α- and β-labeled octanoic acids.

EXPERIMENTAL

The preparation of the carboxyl-labeled acids has been described (2). α-Labeled octanoic acid was prepared as follows: $CH_3(CH_2)_5C^{14}OOH$ was reduced to n-heptyl alcohol by means of $AlLiH_4$ in anhydrous ether (7) and converted to the bromide via HBr and H_2SO_4 (8). The Grignard reagent was prepared and treated with CO_2, and the $CH_3(CH_2)_5C^{14}H_2-COOH$ was obtained in the usual manner. β-Labeled octanoic acid was synthesized by converting $CH_3(CH_2)_4C^{14}OOH$ to the alcohol (via $AlLiH_4$), which was then brominated and treated with malonic ester (9). The

$CH_3(CH_2)_4C^{14}H_2CH_2COOH$ was obtained by heat decarboxylation of the substituted malonic acid. Purification was effected by means of steam distillation and extractions.

The tissue incubations were conducted by the Warburg technique previously described (2). Respired $C^{14}O_2$ and the activity of the carboxyl group of the acetoacetic acid were determined (2). Decarboxylation of the acetoacetic acid was carried out in 125 ml. Warburg flasks with precautions against contamination by extraneous CO_2 or $C^{14}O_2$. An aliquot of the medium was used for the determination of the carbonyl activity as follows: After the addition of a known amount of non-labeled acetone, the mixture was allowed to stand overnight at room temperature and then neutralized to phenol red. The acetone was distilled in an all-glass still into a 25 ml. volumetric flask. After dilution to the mark, an aliquot was used for determination of total acetone (10) and the remainder was treated with 2,4-dinitrophenylhydrazine. The hydrazone was washed three times with water and then recrystallized from ethyl alcohol. Activity measurements were made by plating and counting the hydrazone directly (see footnote to Table I).

Studies were conducted with rat liver and kidney slices, but the former tissue was studied more extensively than the latter. Experimental data pertaining to the various experiments are given in the foot-notes of the proper tables.

RESULTS AND DISCUSSION

The data in Table I give the results of experiments with liver slices and carboxyl-labeled acids and show that the acetoacetic acid formed from either odd or even numbered acids was asymmetrically labeled in the carboxyl and carbonyl groups. Furthermore, the degree of asymmetry was dependent upon the length of the carbon chain of the substrate from which the radiocarbon was derived. Thus, the $C^{14}O:C^{14}OOH$ ratio of the acetoacetic acid formed from $CH_3(CH_2)_4C^{14}OOH$ had a value of approximately 0.48, whereas that formed from lauric acid ($—C^{14}OOH$) had a value of 0.95. The ratio in the case of octanoic acid was 0.74, which is in close agreement with the values reported by Buchanan et al. (4), Weinhouse et al. (1), and Crandall and Gurin (5).[1] The latter investigators also reported that the acetoacetic acid formed from carboxyl-labeled hexanoic acid had a slightly lower $C^{14}O:C^{14}OOH$ ratio than that derived from octanoic acid.

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[1] As pointed out in the foot-note to Table I, the activity of the acetone moiety of the acetoacetic acid was determined by counting the acetone-2,4-dinitrophenylhydrazone directly. This procedure has been found to give results which are approximately 1.1 times too high.

The results obtained with the acids of an odd number of carbons indicate that these acids, like those of an even number of carbons, give rise to 2-carbon fragments when metabolized. These fragments are then incor-

TABLE I

Conversion of $RC^{14}OOH$ to $C^{14}O_2$ and $CH_3C^{14}OCH_2C^{14}OOH$ by Rat Liver Slices

Experiment No.†	Substrate	Total activity per flask	Conversion to $C^{14}O_2$ (A)	Conversion to aceto-acetate carbonyl (B)	Conversion to aceto-acetate carboxyl (C)	Total conversion to aceto-acetate (D)	A+D	$\frac{D}{A}$	$\frac{B}{C}$
		c.p.m.‡	c.p.m.‡	c.p.m.‡	c.p.m.‡	c.p.m.‡	c.p.m.‡		
152	$CH_3(CH_2)_3\overset{*}{C}OOH$	55,200	10,000	1230	3,990	5,220	15,220	0.52	0.31
	$CH_3(CH_2)_4\overset{*}{C}OOH$	54,700	5,740	4120	8,710	12,830	18,570	2.24	0.47
	$CH_3(CH_2)_5\overset{*}{C}OOH$	46,300	7,200	1580	2,970	4,550	11,750	0.63	0.53
	$CH_3(CH_2)_6\overset{*}{C}OOH$	45,500	3,690	3700	5,020	8,720	12,410	2.36	0.74
	$CH_3(CH_2)_7\overset{*}{C}OOH$	55,700	5,760	2980	3,900	6,880	12,640	1.19	0.76
155	$CH_3(CH_2)_{10}\overset{*}{C}OOH$	87,600	8,060	4590	4,810	9,300	17,360	1.15	0.95
153	$CH_3(CH_2)_3\overset{*}{C}OOH$	68,200	9,890	1220	4,450	5,670	15,560	0.57	0.27
	$CH_3(CH_2)_4\overset{*}{C}OOH$	60,500	7,030	5050	10,800	15,850	22,880	2.26	0.47
	$CH_3(CH_2)_5\overset{*}{C}OOH$	66,000	9,590	2900	4,850	7,750	17,340	0.81	0.60
	$CH_3(CH_2)_6\overset{*}{C}OOH$	76,100	5,800	6420	8,640	15,060	20,860	2.60	0.74
	$CH_3(CH_2)_7\overset{*}{C}OOH$	83,100	7,720	4139	4,790	8,929	16,649	1.15	0.86
160	$CH_3(CH_2)_{10}\overset{*}{C}OOH$	46,200	3,850	2350	2,440	4,790	8,640	1.24	0.96

† Each flask contained 20 cc. of Ca-free Ringer-phosphate solution at pH 7.0. Incubation period 60 minutes at 38° in an O_2 gas phase. Approximately 0.3 gm. (dry weight) of liver slices per flask used in all experiments except Experiment 160 in which 0.49 gm. (dry weight) was used. All substrates were present at a concentration of 0.0013 M.

‡ Counts per minute per gm. of dry tissue. Columns (A) and (C) were obtained by counting $BaC^{14}O_3$, whereas column (B) was obtained by counting the 2,4-dinitrophenylhydrazone of acetone. Subsequent studies have shown that the latter activities are high by a factor of about 1.1. To facilitate comparisons, all values have been corrected for differences between specific activities of the various substrates by using octanoic acid as the standard and making all other values relative to this.

porated into acetoacetic acid by a mechanism which must be similar to that involved when the fragments are derived from even numbered acids. As shown by the data in Table I, the ratios in the case of hexanoic and hep-

tanoic acids are fairly similar, as are those in the case of octanoic and nonanoic acids. This phenomenon suggests (a) that the terminal 3-carbon fragments that would be formed from odd numbered acids by β oxidation could be degraded to terminal 2-carbon fragments similar to those arising from the even numbered acids, or (b) that terminal 2-carbon fragments from normal substrates present in the slices can interact with 2-carbon fragments derived from the odd numbered acids. The over-all effect was that an odd numbered acid behaved as though the number of 2-carbon fragments derived from it was equal to those available from the next lower adjacent *even numbered* acid.

The data presented in Table II show that, in accord with the findings of Crandall and Gurin (5), acetoacetic acid is not formed by alternate oxidation of even numbered acids. The $C^{14}O : C^{14}OOH$ ratios for the acetoacetic acid derived from carboxyl- and β-labeled octanoic acid were the same. Crandall and Gurin (5) reported a slightly lower value in the case of the β-labeled acid. The results obtained with the α-labeled acid are those expected on the basis of acetoacetic acid formation by recondensation of 2-carbon fragments. Furthermore, like the results obtained by Weinhouse *et al.* (11), these, too, indicate that there is little, if any, equilibration between the 2 carbons of the 2-carbon fragments. The low $C^{14}O_2$ production from the α-labeled acid as compared with that from the carboxyl-labeled acid may be due to the fact that the methylene carbons of ($-CH_2CO-$) fragments must make at least one complete cycle through the tricarboxylic acid cycle before any of the carbon can be obtained as CO_2. The carbon which is not evolved as CO_2 during the second cycle will require a number of passages through the cycle before most of it can be oxidized to CO_2. This means an over-all effect of having an appreciable amount of this carbon incorporated in various metabolic intermediates at any given time.[2]

From a comparison of the data given in Tables I and III, it can be seen that not only was less acetoacetic acid derived from the various substrates by kidney slices than by liver slices, but the $C^{14}O : C^{14}OOH$ ratios were much lower in the case of the former tissue. That this is not due to alternate oxidation in the case of kidney is shown by the fairly similar $C^{14}O : C^{14}OOH$ ratios obtained with carboxyl- and β-labeled octanoic acids. These results indicate that acetoacetic acid is not an intermediate in the oxidation of fatty acids by kidney tissue. When the values for the total radiocarbon converted to $C^{14}O_2$ and $CH_3C^{14}OCH_2C^{14}OOH$ are considered, it appears that liver metabolized more substrate than did kidney only when

[2] The number of cycles necessary to liberate all of these labeled methylene carbons as CO_2 depends upon their distribution in the various positions of the intermediates of the tricarboxylic acid cycle.

TABLE II

Conversion of Carboxyl-, α-, and β-Labeled Octanoic Acids to $C^{14}O_2$ and Acetoacetate

Experiment No.‡	Tissue	Weight of dry tissue	Substrate and concentration, R = CH₃(CH₂)ₙ—	M	Total activity per flask	Conversion to $C^{14}O_2$ (A)	Conversion to acetone moiety of acetoacetate (B)	Conversion to acetoacetate carboxyl (C)	Total conversion to acetoacetate (D)	A + D	D/A	B/C
		gm.		M	c.p.m.‡	c.p.m.‡	c.p.m.‡	c.p.m.‡	c.p.m.‡	c.p.m.‡		
164	Liver	0.2428	RCH₂CH₂COOH*	0.001	61,300	4,370	5,610	9,370	14,980	19,350	3.42	0.60
		0.2985	RCH₂CH₂*COOH	0.001	49,900	1,730	11,780	100	11,880	13,610	6.86	117.8
		0.2451	RCH₂*CH₂COOH	0.001	60,600	5,000	5,650	9,350	15,000	20,000	3.00	0.65
	Kidney	0.2321	RCH₂CH₂COOH*	0.001	64,200	21,700	780	3,370	4,150	25,850	0.19	0.23
		0.2373	RCH₂CH₂*COOH	0.001	63,000	11,700	1,700	85	1,785	13,485	0.15	20.0
		0.2354	RCH₂*CH₂COOH	0.001	63,400	18,000	560	2,360	2,920	20,920	0.16	0.24
158	Liver	0.3196	RCH₂CH₂COOH*	0.0013	58,700	8,310	6,000	11,200	17,200	25,510	2.07	0.54
		0.3300	RCH₂CH₂*COOH	0.0013	60,200	4,020	15,020	510	15,530	19,550	3.86	29.5
		0.3498	RCH₂*CH₂COOH	0.00074	31,500	5,500	3,850	6,450	10,300	15,800	1.87	0.60
160	"	0.5347	RCH₂CH₂COOH*	0.0013	36,400	5,680	4,520	6,220	10,740	16,420	1.90	0.73
		0.4630	RCH₂CH₂*COOH	0.00074	23,900	4,340	2,780	3,900	6,680	11,020	1.54	0.71
159	Kidney	0.2629	RCH₂CH₂COOH*	0.0013	73,700	24,400	1,280	3,220	4,500	28,900	0.18	0.40
		0.2249	RCH₂CH₂*COOH	0.00074	48,900	21,100	450	1,590	2,040	23,140	0.10	0.28

† Conditions of incubation given in foot-note to Table I. Incubation time for all experiments except Experiment 164, 60 minutes; that of Experiment 164, 45 minutes.

‡ Counts per minute per gm. of dry tissue.

the fatty acid was of an even number of carbons. As pointed out earlier (2, 11), from the standpoint of complete oxidation, kidney metabolizes more fatty acid than does liver.

The mechanism of acetoacetic acid formation by liver advanced by Crandall *et al.* (5, 6) to explain the asymmetry of the $C^{14}O:C^{14}OOH$ ratio can be applied to the data in this paper. Their basic concept is that during the β oxidation of fatty acids all of the 2-carbon fragments except the terminal one (CH_3CO-) are alike, namely ($-CH_2CO-$). The (CH_3-CO-) fragments are converted to the "active acetate" type only to a

TABLE III

Conversion of $RC^{14}OOH$ to $C^{14}O_2$ and $CH_3C^{14}OCH_2C^{14}OOH$ by Rat Kidney Slices

Experiment No.†	Substrate	Total activity per flask	Conversion to $C^{14}O_2$ (A)	Conversion to acetoacetate carbonyl (B)	Conversion to acetoacetate carboxyl (C)	Total conversion to acetoacetate (D)	$A + D$	$\dfrac{D}{A}$	$\dfrac{B}{C}$
		c.p.m.‡	*c.p.m.‡*	*c.p.m.‡*	*c.p.m.‡*	*c.p.m.‡*	*c.p.m.‡*		
156	$CH_3(CH_2)_3\overset{*}{C}OOH$	71,600	13,200	65	965	1030	14,230	0.08	0.07
	$CH_3(CH_2)_4\overset{*}{C}OOH$	66,100	12,400	270	1570	1840	14,240	0.15	0.17
	$CH_3(CH_2)_5\overset{*}{C}OOH$	82,000	13,700		1040	1040	14,740	0.08	
	$CH_3(CH_2)_6\overset{*}{C}OOH$	69,900	11,610	420	1950	2370	13,980	0.20	0.21
	$CH_3(CH_2)_7\overset{*}{C}OOH$	73,200	13,800	90	1540	1630	15,430	0.12	0.06

† Each flask contained 20 cc. of Ca-free Ringer-phosphate solution at pH 7.0. Incubation period 30 minutes at 38° in an O_2 gas phase. Approximately 0.3 gm. (dry weight) of kidney slices per flask. All substrates were present at a concentration of 0.0013 M.

‡ Counts per minute per gm. of dry tissue.

limited extent, whereas the ($-CH_2CO-$) type can easily enter into acetoacetate synthesis as either the carbonyl or the carboxyl moiety. Thus, the terminal group of a carboxyl-labeled acid acts as a non-labeled diluent as far as the carbonyl carbon is concerned. The shorter the carbon chain of the fatty acid, the fewer the ($-CH_2CO-$) groups formed relative to the (CH_3CO-) groups, with the consequent lowering of the $C^{14}O:C^{14}OOH$ ratio. In conformity with this, the ratios increased from 0.30 to 0.95 for the acids from pentanoic through dodecanoic, inclusive.

There are several interesting points which require attention. One is the fact that the studies of Crandall *et al.* (5, 6) were carried out with *washed* homogenates of liver, whereas liver slices were used in the present studies and in those reported by Weinhouse *et al.* (1) and Buchanan *et al.* (4). In the case of the homogenates, little endogenous metabolism occurred,

while a great deal occurred with the liver slices. In spite of these differences, comparable results were obtained by all investigators. It might well have been expected that in the liver slices the large number of both (CH_3CO—) and (—CH_2CO—) groups being formed continuously from normal substrate would negate the influence of the relative proportion of them formed from the added substrate. This was not the case, however, for, as the results indicate, either there was little interaction between fragments from the two sources or the extent of interaction was conditioned by the length of the carbon chain of the added substrate. If the normal substrates undergoing metabolism were mainly long chain fatty acids and pyruvic acid, the (—CH_2CO—) type of fragment would predominate. These would augment the formation of "symmetrical" acetoacetic acid. However, liver appears to have a rather constant rate of complete fatty acid oxidation (1, 2) and it seems probable that the added substrates are oxidized in preference to the normal lipides. This would aid in obtaining the "terminal group influence" of the added substrate.

The nature of the carbonyl moiety of acetoacetic acid formed from odd numbered acids is also interesting. Since the apparent supply of terminal 2-carbon fragments is influenced by the chain length of these acids, it may indicate that conversion of the 3-carbon group (CH_3CH_2CO—) to the (CH_3CO—) type occurs to a certain extent. That such a conversion would not have to be extensive is indicated by the fact that the odd numbered acids give rise to relatively little acetoacetic acid.

SUMMARY

When incubated with liver slices, both odd numbered and even numbered acids ($RC^{14}OOH$) gave rise to asymmetrically labeled acetoacetic acid. The degree of asymmetry was conditioned by the length of the carbon chain of the substrate.

Carboxyl- and β-labeled octanoic acids gave rise to acetoacetic acid with the same $C^{14}O:C^{14}OOH$ ratio. α-Labeled octanoic acid gave rise to acetoacetic acid which contained radiocarbon almost exclusively in the acetone moiety.

The acetoacetic acid formed by kidney slices had a lower $C^{14}O:C^{14}OOH$ ratio generally than did that formed from these substrates by liver slices.

The results are discussed from the standpoint of the influence of the relative number of terminal (CH_3CO—) groups and (—CH_2CO—) groups on the symmetry of the acetoacetic acid produced.

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INFLUENCE OF SULFHYDRYL REAGENTS ON THE CYTOCHROME c-CYTOCHROME OXIDASE SYSTEM*

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Earlier papers (1, 2) from this laboratory demonstrated that basic phenylmercuric nitrate depressed the cytochrome c-cytochrome oxidase system, determined manometrically, as well as yeast respiration (3) and bacterial growth (4), and that protection against these depressions could be afforded by sulfhydryl compounds (2–4) which, however, would not reverse the depression once it was established. It seemed possible that these effects might be accounted for by reaction with essential —SH groups. However, Barron and Singer (5, 6) reported that cytochrome oxidase activity was unaffected by p-chloromercuribenzoate, trivalent arsenicals, and iodoacetamide, and Gordon and Quastel (7) also noted a lack of response to mapharside. On the basis of these results the enzyme has been regarded as not being sulfhydryl-dependent. Phenylmercuric nitrate thus appeared to be non-specific as compared with p-chloromercuribenzoate, which accords with the findings of Thimann and Bonner on the growth and respiration of isolated plant parts (8).

It seemed advisable to compare directly the action of phenylmercuric nitrate on the cytochrome oxidase system with other RHgX compounds (phenylmercuric hydroxide and phenylmercuric chloride) and especially with p-chloromercuribenzoate and p-aminophenylarsine oxide which have been regarded as highly selective in their combinations with —SH groups (5, 9, 10). A few experiments were conducted with less specific iodoacetate and potassium ferricyanide. Manometric observations were supplemented with the spectrophotometric studies of the effects of these inhibitors on cytochrome c and on the rate of oxidation of reduced cytochrome c by cytochrome oxidase.

EXPERIMENTAL

Manometric Methods—The activity of the cytochrome oxidase system was measured in Warburg manometers at 37.5°. With ascorbic acid as the substrate, we followed the method of Schneider and Potter (11), employing an excess of cytochrome c. The center well of the respirometer con-

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tained 0.2 ml. of 2 N sodium hydroxide. The main chamber contained 1.0 ml. of 2.4×10^{-4} M cytochrome *c* (molecular weight 16,500), 0.3 ml. of freshly prepared 0.114 M ascorbic acid in 0.1 M phosphate buffer, pH 7.4, 0.3 ml. of 4×10^{-3} M aluminum chloride, 0.4 ml. of oxidase-containing extract, and 1.0 ml. of 0.1 M phosphate buffer, pH 7.4. In the experimental flasks 0.5 ml. of buffer was replaced with the desired concentrations of inhibitor dissolved in buffer. In protection or reversal experiments a further 0.5 ml. of buffer was replaced by the desired concentration of sulfhydryl compound. The inhibitor or sulfhydryl compound was placed in the main chamber or side arm as desired. In the experiments in which cytochrome *c* was previously treated with the inhibitors, the amount of cytochrome *c* was reduced to 2.48×10^{-8} mole per flask to make it the limiting factor rather than the oxidase. Preliminary experiments showed the limiting concentration to be 3.5×10^{-8} mole per flask.

With hydroquinone as substrate essentially the procedure of Schultze (12) was employed. The center well held 0.2 ml. of 2 N sodium hydroxide. The main chamber contained 1.0 ml. of 2.4×10^{-4} M cytochrome *c*, 0.3 ml. of 0.18 M semicarbazide hydrochloride in buffer, 0.4 ml. of oxidase-containing extract, and 1.0 ml. of M/15 phosphate buffer, pH 7.1. The side arm contained 0.3 ml. of 0.21 M resublimed hydroquinone in buffer which was tipped in after the 15-minute equilibrium period. The inhibitor or sulfhydryl compound replaced the buffer when necessary, as in the experiments with ascorbic acid. All tests were accompanied by controls for autoxidation of substrate and for oxidation of substrate by enzyme in the presence of cytochrome *c*.

Spectrophotometric Methods—The Beckman model DU spectrophotometer was used. In studying the effects of inhibitors on the absorption spectra of cytochrome *c*, 1 ml. of a solution containing 2.0 to 3.5 mg. of cytochrome *c* and 3 ml. of phosphate buffer were added to the inhibitor solutions to give a ratio which was the same as that found in the manometric studies to give 100 per cent inhibition of the complete system, with the exception of the arsenical which was used in a ratio that gave 76 per cent depression manometrically. The following weights of inhibitors in mg. per mg. of cytochrome *c* were employed: phenylmercuric nitrate, 0.12; phenylmercuric hydroxide, 0.11; *p*-chloromercuribenzoate, 0.13; *p*-aminophenylarsine oxide, 0.67. After incubation of the mixtures at 37° for 1 hour, the solutions were oxidized with 1 ml. of 0.01 M potassium ferricyanide and diluted to 10 ml. in volumetric flasks. The oxidized spectrum was then taken, after which a few grains of solid sodium dithionite were added to obtain the reduced spectrum. Control solutions were given the same treatment without addition of sulfhydryl reagent.

For the determination of oxidation rate, mixtures of cytochrome *c* and

sulfhydryl reagent were prepared as above (except that the ratio of cyto-
chrome c to arsenical was 1:0.15, which gave 50 per cent depression mano-
metrically), incubated for 1 hour, and aliquots containing from 0.96 to 1.10
mg. of cytochrome c were placed in the absorption cells with 1 ml. of phos-
phate buffer and diluted with water to 2.98 ml. The cell contents were
shaken with enough solid sodium dithionite to give 80 to 90 per cent reduc-
tion (avoid excess of reducing agent) and read, along with controls, at 550 mμ
with a 0.01 mm. slit width. To each cell was added 0.02 ml. (0.5 mg.) of
cytochrome oxidase, and, after shaking, readings were taken every 10
seconds for 90 seconds. The concentration of reduced cytochrome c at
any time was calculated according to the following equation (13).[1]

$$(\text{Cytochrome Fe}^{++}) = \frac{\log I_0/I - \log I_0/I_{ox.}}{(\alpha_r. - \alpha_{ox.})l}$$

In dialysis studies a cytochrome c-phenylmercuric nitrate mixture (1:0.12
mg.) treated as above was dialyzed against glass-distilled water for 3 days,
the dialysate was evaporated to dryness and ashed, and the mercury was
determined by White's procedure (14). The purity of the dialyzed cyto-
chrome c was checked spectrophotometrically, its rate of oxidation by oxi-
dase was compared with that of untreated cytochrome c, and its efficiency
as a catalyst in the enzyme system was compared manometrically with un-
treated cytochrome c.

For investigations of cytochrome oxidase inhibition, the oxidase was
treated with inhibitors at 4°. A series of mixtures was studied in which the
ratio of oxidase to inhibitor was varied. In all cases 0.50 mg. of oxidase
was introduced into the spectrophotometer cell.

Materials—Cytochrome c was prepared from beef heart by the method
of Keilin and Hartree (15) except that it was dialyzed against glass-distilled
water instead of 1 per cent sodium chloride. In most cases it was further
purified by repetition of the ammonium sulfate and trichloroacetic acid
precipitation. By colorimetric analysis with α, α'-dipyridyl and spectro-
scopic analysis (16), different preparations varied in purity from 80 to 97
per cent, based on an iron content of 0.34 per cent (Keilin and Hartree
(15)).

Rat heart extract prepared in phosphate buffer, pH 7.4, was the source
of cytochrome oxidase. All extracts were prepared on the day of use and
varied in solid content from 24 to 26 mg. per ml.

Basic phenylmercuric nitrate and phenylmercuric hydroxide were ob-

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[1] $\alpha_r.$ and $\alpha_{ox.}$ = extinction coefficients of reduced and oxidized cytochrome c re-
spectively; (Cytochrome Fe^{++}) = concentration of reduced cytochrome c; l =
length of absorption tube; log I_0/I = extinction of mixture of oxidized and reduced
cytochrome c; log $I_0/I_{ox.}$ = extinction of completely oxidized cytochrome c.

tained from The Hamilton Laboratories, Inc., and were prepared as stock solutions of 6.3 × 10⁻⁴ M and 2.7 × 10⁻³ M, respectively. *p*-Chloromercuribenzoic acid (Schaefer Laboratories) was dissolved in the ratio of 10 mg. per 5 ml. of water containing 0.05 ml. of 2 N sodium hydroxide, and *p*-aminophenylarsine oxide (Bios Laboratories, Inc.) was prepared as an aqueous suspension, 20 mg. per ml. All solutions were diluted with 0.1 M phosphate buffer, pH 7.4, to obtain the desired experimental concentrations. L-Cysteine hydrochloride and semicarbazide hydrochloride were
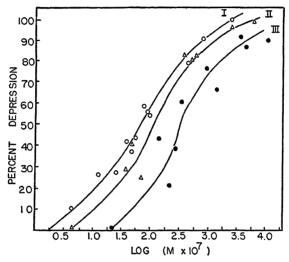
Fig. 1. Depression of cytochrome oxidase system (manometric) by Curve I, basic phenylmercuric nitrate; Curve II, phenylmercuric hydroxide; Curve III, *p*-chloromercuribenzoate.

brought to the desired pH with dilute sodium hydroxide. Glass-distilled water was used in all procedures.

Results

Preliminary manometric experiments confirmed the previous results (1) that basic phenylmercuric nitrate depressed the oxygen uptake of the cytochrome c-cytochrome oxidase system with either ascorbic acid or hydroquinone as substrate. Phenylmercuric hydroxide and *p*-chloromercuribenzoate depressed the system as well, and a comparison of the effects of these reagents with phenylmercuric nitrate, with ascorbic acid as substrate, is given in Fig. 1. Results were similar with hydroquinone. A limited number of experiments with phenylmercuric chloride showed it to behave

similarly to phenylmercuric nitrate and hydroxide; *e.g.*, at 8.3 × 10⁻⁵ M it produced 84 per cent depression with ascorbic acid and 76 per cent depression with hydroquinone at the end of 2 hours.

p-Aminophenylarsine oxide also depressed the system with both substrates but considerably higher concentrations were necessary than in the case of the mercurials. These concentrations brought the arsenical to the limit of its solubility; thus some experiments had to be performed with the material partly in suspension. This produced a greater scatter in the experimental values which did not justify construction of a curve, although the results gave the same general pattern as those with the mercurials, with considerable displacement to the right. *p*-Aminophenylarsine oxide of 5 × 10⁻³ M and above produced depressions of 75 to 100 per cent, and approximately 50 per cent depression was given by 1.7 × 10⁻³ M.

Cysteine, when present from the beginning of the manometric experiments, protected the system against the mercury and arsenic compounds, as it had against phenylmercuric nitrate (2), but was unable to reverse the depression effectively when added during the course of the experiment. Cysteine, 10⁻³ M, gave 100 per cent protection against 5 × 10⁻⁴ M *p*-chloromercuribenzoate and 70 to 80 per cent protection against 2.5 × 10⁻⁴ M phenylmercuric hydroxide. Cysteine, 10⁻² M, completely protected the system against 5 × 10⁻³ M *p*-aminophenylarsine oxide. As shown earlier for phenylmercuric nitrate (2), no amino acids containing reactive groups other than sulfhydryl (cystine, methionine, tyrosine, serine, arginine, proline, lysine, phenylalanine, aspartic acid, and tryptophan) protected the system against the inhibitors. The protection apparently afforded by histidine with ascorbic acid as substrate (2) was found in the present experiments to be false and due to the ascorbic acid-catalyzed oxidation of histidine (17).
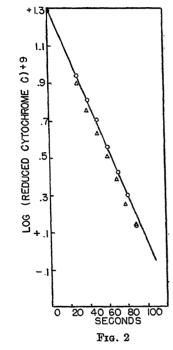
Iodoacetate was a poor and somewhat erratic inhibitor, producing from 0 to 13 per cent depression at 10⁻³ M manometrically. Potassium ferricyanide, observed in a few experiments, was also of low efficiency as an inhibitor, completely inhibiting only at the very high concentration of 0.1 M and causing an average of 20 per cent inhibition at 0.01 M. These compounds were not studied spectrophotometrically.

Experiments were also performed in which the substrates (ascorbic acid and hydroquinone) were subjected to the inhibitors (phenylmercuric nitrate, phenylmercuric hydroxide, phenylmercuric chloride, *p*-chloromercuribenzoate, and *p*-aminophenylarsine oxide) in the absence of the enzyme. None of the compounds in concentrations which inhibited the enzyme depressed the autoxidation rate of the substrate alone.

Forty spectrophotometric studies with four different cytochrome *c* preparations revealed that none of the mercurial or arsenical compounds altered

the position or extinction of the normal maxima of the oxidized, reduced, or mixed valence forms.

The rate of oxidation of reduced cytochrome *c* was not affected by incubation with the inhibitors, as is illustrated by the typical experiment with *p*-chloromercuribenzoate given in Fig. 2. Likewise, the rate of oxidation
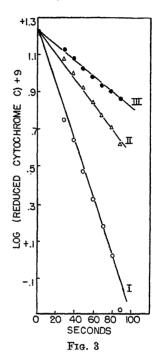
FIG. 2. Effect of *p*-chloromercuribenzoate treatment of cytochrome *c* on its rate of oxidation by cytochrome oxidase. O, untreated; △, treated (1 mg. of cytochrome *c* to 0.13 mg. of *p*-chloromercuribenzoate).

FIG. 3. Effect of *p*-chloromercuribenzoate treatment of cytochrome oxidase on its rate of oxidation of cytochrome *c*. Curve I, untreated; Curve II, treated (1 mg. of oxidase to 0.12 mg. of *p*-chloromercuribenzoate); Curve III, treated (1 mg. of oxidase to 0.16 mg. of *p*-chloromercuribenzoate).

of cytochrome *c*, treated with phenylmercuric nitrate and dialyzed free of mercury, was unaffected; the mercury was recovered quantitatively in the dialysate. Such treatment also did not affect the ability of cytochrome *c* to function efficiently in the oxidation of ascorbic acid by cytochrome oxidase manometrically when cytochrome *c* was limiting.

Incubation of the cytochrome oxidase preparations with the inhibitors impaired the ability of the oxidase to oxidize reduced cytochrome *c*, as is

illustrated by the results of an experiment with p-chloromercuribenzoate shown in Fig. 3. Table I summarizes the effects of incubating cytochrome oxidase with varying amounts of the inhibitors and compares the degree of depression obtained by manometric and spectrophotometric procedures.

TABLE I

Inhibitory Activity of —SH Reagents on Cytochrome Oxidase

Ratio, oxidase to reagent*	No. of experiments	Average per cent depression	
		Manom- eter	Spectro- photom- eter
1:0.04 basic phenylmercuric nitrate	6	50	51
1:0.02 phenylmercuric hydroxide	3	0	0
1:0.04 " "	5	50	46
1:0.04 p-chloromercuribenzoic acid	4	20	0
1:0.08 " "	2	33	0
1:0.12 " "	4	43	55
1:0.16 " "	4	50	67
1:1.20 p-aminophenylarsine oxide	5		11
1:2.20 " "	4	39	35

* Amounts of oxidase and reagent given in mg.

DISCUSSION

The spectra provide no evidence of a reaction between cytochrome c and the inhibitors, and it was possible to dialyze phenylmercuric nitrate quantitatively from cytochrome c with no impairment of the functioning of the enzyme in the manometric system. Manometric experiments showed that the inhibitors do not affect the autoxidation rate of the substrate. Treatment of the cytochrome oxidase preparations with the inhibitors depressed the ability of the preparations to oxidize reduced cytochrome c (spectrophotometrically) or to oxidize ascorbic acid or hydroquinone through cytochrome c (manometrically). Cytochrome oxidase would therefore seem to be the site of activity of the inhibitors.

The relative inhibiting activities of the heavy metal compounds are shown by the following approximate concentrations which produced a 50 per cent depression of the activity of the cytochrome c-cytochrome oxidase system as determined manometrically with ascorbic acid as substrate (hydroquinone gave comparable results): basic phenylmercuric nitrate, 6.3 $\times$ 10^{-6} M; phenylmercuric hydroxide, 1.2 $\times$ 10^{-5} M; p-chloromercuribenzoate, 3.2 $\times$ 10^{-5} M; and p-aminophenylarsine oxide, 1.7 $\times$ 10^{-3} M. Referred to phenylmercuric nitrate as having an inhibitory effectiveness of 1, the following approximate ratings are obtained: phenylmercuric hydroxide, 0.5;

p-chloromercuribenzoate, 0.2; *p*-aminophenylarsine oxide, 0.004. The depressions obtained spectrophotometrically are generally in good agreement with those found manometrically, as shown in Table I. However, inhibition with low concentrations of *p*-chloromercuribenzoate is evident with the manometric but not with the spectrophotometric technique; at higher concentrations depression is obtained by both methods.

Our results with *p*-chloromercuribenzoate are at variance with those of Barron and Singer (5). Possibly this is due to the fact that these workers employed the spectrophotometric method which, according to our data (Table I), does not indicate depressions with lower concentrations of this inhibitor, while the manometric method does. Coincidental with our work, Slater (18) has also found that cytochrome oxidase is somewhat inhibited by *p*-chloromercuribenzoate. The previously reported lack of response of the enzyme to arsenicals (5–7) may be related to our observations of the relatively low sensitivity of the enzyme to *p*-aminophenylarsine oxide.

We do not feel that our results necessarily indicate that cytochrome oxidase is a sulfhydryl-dependent enzyme, especially in view of our failure to reverse the inhibitions by subsequent addition of cysteine. The reversibility of the enzyme-heavy metal reagent mercaptide by addition of an excess of simple mercaptan is usually regarded as characteristic (10, 19). Rather, it would seem that the results are due to the non-sulfhydryl-specific nature of the reagents. While the present experiments were in progress, other evidence has appeared which indicates the non-specificity of the mercurial reagents. This includes Slater's (18) and our (20) observations on the greater sensitivity of the succinoxidase than of the succinic dehydrogenase system and Hellerman's observations (21) on the response of D-amino acid oxidase.

The nature of the non-specific reaction is not clear from the present results. The inability of amino acids containing reactive groups other than —SH to protect against the inhibitors leaves us without evidence as to what groups of the protein might combine with the inhibitors. It is possible that the effect is of a more general nature upon the physical properties of the system, since the cytochrome oxidase preparation is of a particulate nature. Keilin and Hartree (22) and Slater (18, 23) have recently emphasized the importance of this type of effect on the succinoxidase system.

SUMMARY

1. The oxygen uptake of the cytochrome *c*-cytochrome oxidase system, with ascorbic acid or hydroquinone as substrate, is inhibited by basic phenylmercuric nitrate, phenylmercuric hydroxide, phenylmercuric chloride, *p*-chloromercuribenzoate, and *p*-aminophenylarsine oxide, the last compound being the least effective. Iodoacetate and potassium ferri-

cyanide are poor inhibitors. Cysteine protects against but does not reverse the effects of the metal compounds.

2. The inhibitors do not affect the autoxidation rate of the substrates.

3. Treatment of cytochrome c with the mercury and arsenic compounds does not affect the absorption spectra of the oxidized, reduced, or mixed forms, does not affect its oxidation by cytochrome oxidase, and does not impair its function as a catalyst in the cytochrome c-cytochrome oxidase system.

4. Crude cytochrome oxidase can be inactivated by treatment with the mercury and arsenic compounds as shown by its inability to oxidize reduced cytochrome c. The spectrophotometric data compare satisfactorily with the manometric.

5. The inhibitory effects seem to be exerted upon the cytochrome oxidase portion of the system and are probably due to non-sulfhydryl specific action of the inhibitors.

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STUDIES ON CHOLINESTERASE

VII. THE ACTIVE SURFACE OF ACETYLCHOLINE ESTERASE DERIVED FROM EFFECTS OF pH ON INHIBITORS*

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In previous investigations the kinetics of the inhibition of acetylcholine esterase were studied with two reversible inhibitors, prostigmine and eserine, and two irreversible inhibitors, diisopropyl fluorophosphate (DFP) and tetraethyl pyrophosphate (TEPP) (1, 2). One common feature resulted from the data obtained; namely, that all four inhibitors appear to act upon the same groups in acetylcholine esterase at which acetylcholine is hydrolyzed. The competition between inhibitor and substrate for the same surface region is suggested by two types of observations: (*a*) The sequence in which inhibitor and substrate come in contact with the enzyme affects the course of hydrolysis in all cases. This suggests that the substrate has a protecting effect by competing with all four inhibitors for the active center. (*b*) When a concentrated enzyme solution is incubated with prostigmine prior to its incubation with DFP and later diluted, complete protection against DFP may be demonstrated, suggesting that formation of the prostigmine-enzyme complex may occur at the same functional groups as those involved in the reaction of DFP with the enzyme.

In spite of this common feature, considerable difference exists between the various inhibitors. When, for example, the activity-substrate concentration relationship was tested in the presence of prostigmine, the optimum substrate concentration was progressively higher with increasing inhibitor concentration. This shift was also evident in the case of eserine, but smaller; none was observed with DFP and TEPP.

It appeared of interest to obtain more information about the factors affecting the mode of combination of the enzyme with the four inhibitors. Studies of the effects of pH changes are described in this paper.

Methods

The enzyme used throughout was highly purified acetylcholine esterase prepared from the electric tissue of *Electrophorus electricus* according to

the method previously described (3). The hydrolysis of acetylcholine was determined in most cases with the colorimetric method (4), except in the dilution experiments with TEPP, in which the manometric technique was applied. All experiments were carried out at room temperature (20–23°).

For pH 8.5 and below phosphate buffer of the following composition was used: 0.1 M Na$_2$HPO$_4$, adjusted to the pH desired, 0.1 M NaCl, 0.0008 M MgCl$_2$, and 0.1 per cent gelatin. Above pH 8.5 the corresponding 0.1 M borate buffer was used. Gelatin was employed to increase the stability of the highly diluted purified enzyme. The acetylcholine concentration was usually 3 to 4 × 10^{-3} M final concentration, which is close to the optimum.

The inhibitors used were prostigmine bromide, Hoffman-La Roche, eserine sulfate, Merck, and TEPP, Monsanto, of 40 per cent purity. Dimethylaminoethyl acetate hydrochloride (5) was recrystallized from a mixture of 1 part of ethanol in 20 parts of ethyl acetate.[1]

Results

Prostigmine—Prostigmine (8.5 × 10^{-7} M) was incubated with acetylcholine esterase for 20 minutes at pH values between 6 and 10. Acetylcholine was then added to start the reaction and after 30 minutes samples were analyzed for the ester. The activity without prostigmine was determined simultaneously. At pH levels above 8 controls were run without the enzyme to correct for non-enzymatic hydrolysis. The correction is significant only above pH 9.

Prostigmine inhibits competitively. When acetylcholine is added after incubation of the enzyme with prostigmine, the substrate shows little competition with the inhibitor in the first few minutes. It takes about 20 to 30 minutes until the new equilibrium is established. The data reported here therefore represent an average of the reaction before and after equilibrium.

The prostigmine cation remains unaltered with pH changes; the protein accepts or donates protons in conformity with the acidity of the medium. These changes in the protein molecule do not markedly affect the inhibition, as illustrated in Fig. 1.

Eserine—Inhibition with eserine (3 × 10^{-7} M) was measured as with prostigmine. As with prostigmine, the competitive inhibition approaches equilibrium slowly; hence the data here reported are again an average of the period before and after equilibrium. Eserine differs from prostigmine in that it exists predominantly as a cation in neutral and acid solution and

, as an uncharged base in alkaline medium. The relative amounts of cation and base at different pH are indicated in Fig. 2, which is a pH titration curve of the sulfate. The abscissa represents the volume of sodium hydroxide but has been recalculated for the per cent of free base, since this is the more pertinent information. Therefore, the scale is not linear. Near the extremes of the curve the per cent of free base must be calculated from the ionization constant of the cation. The constant obtained from this curve, with the concentrations of base and cation but the activity of hydrogen ion, was 8×10^{-9} for an ionic strength of about 0.1. The decrease in inhibition at high pH, compared with the constant inhibition
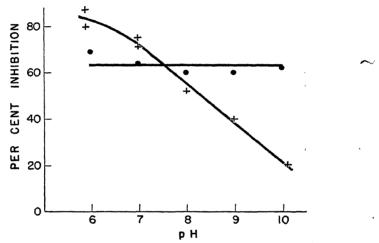
FIG. 1. Inhibition of acetylcholine esterase by prostigmine (●) and eserine (+) as a function of pH.

of prostigmine, as shown in Fig. 1, indicates that the cation of eserine is more potent as an inhibitor than is its uncharged base.

However, the base is also an inhibitor and the entire inhibition at pH 10.1 must be ascribed to this form (Table I). The dissociation constant for the eserine-enzyme complex is given by Augustinsson and Nachmansohn as 1.6×10^{-7}, from which the inhibition corresponding to the low cation concentration at pH 10 can be evaluated. The inhibition so determined is zero.

It may be noted that eserine was stable at pH 10 for the duration of the experiments (60 to 90 minutes). We found no significant decrease of inhibitory action after bringing the solution back to pH 7.0, in spite of the appearance of pink color in concentrated solution. Therefore, any influence of chemical decomposition as a factor in the experiments at high pH may be excluded.
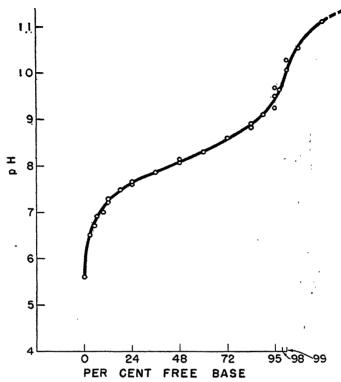
FIG. 2. Titration curve of eserine. The original abscissa of ml. of NaOH was converted into the non-linear scale of free base.

TABLE I

Inhibition of Acetylcholine Esterase by Eserine (3 × 10⁻⁷ M) As Function of pH in Relation to Cation Present

pH	Per cent inhibition	Per cent cation (20°)
5.9	87, 80	99
7	75, 71	92
8	52	44
9	40	12
10.1	20	0.6

Dimethylaminoethyl Acetate—Dimethylaminoethyl acetate (3.2 × 10⁻³ M) was studied as a substrate for acetylcholine esterase at pH levels between 6 and 10. This compound was estimated by the method employed for acetylcholine. At the optimum concentration of acetylcholine and at pH 7 this compound is hydrolyzed about 60 per cent as fast as acetylcholine.

From the titration curve of dimethylaminoethyl acetate the ionization constant was calculated in the same way as for eserine and found to be 5×10^{-8}.

Acetylcholine is hydrolyzed enzymatically at a maximum rate between pH 7 and 9 (6). Since it exists only as cation, the effect of pH must be attributed exclusively to changes of the protein. The rate of hydrolysis of dimethylaminoethyl acetate as a function of pH is shown in Table II. In this case, however, the variations of the ratio of cation to base of the substrate also influence the rate of reaction. To evaluate this second factor separately, the values observed must be corrected for the changing activity of the protein by comparison with the corresponding rates for acetylcholine hydrolysis. For example, the rate of hydrolysis of acetyl-

TABLE II

Hydrolysis of Dimethylaminoethyl Acetate (3.2×10^{-3} M) by Acetylcholine Esterase As Function of pH

Colorimetric determination; 100 Klett units correspond to 1 μM of acetylcholine; temperature 20°.

pH	Rate of hydrolysis	Rate relative to acetylcholine (calculated)	Per cent cation
	Klett units	*Klett units*	
6	50	84	98
7	84	91	92
8	81	81	75
9	34	34	16
10	28	34	1

choline at pH 6 is 60 per cent of the maximum. On the assumption that the protein changes in both cases quantitatively in the same way, the rate of hydrolysis of dimethylaminoethyl acetate at pH 6 may be divided by 0.6. The values so obtained are recorded in Table II.

Again the cation is the more active form, but the neutral molecule is also hydrolyzed, though at a much lower rate. At pH 10 the cationic concentration is 3.2×10^{-5} M. It may be inferred from acetylcholine that this concentration is too low to yield an appreciable rate of hydrolysis for the cationic form. Thus, the hydrolysis at pH 10 must be entirely due to the basic form.

TEPP—The influence of pH on the activity of TEPP was investigated by two types of experimental approach.

In one, concentrated solutions of acetylcholine esterase were incubated for 10 minutes with constant amounts of TEPP buffered at the pH required. The mixture was then sufficiently diluted with gelatin-bicarbon-

ate buffer to render the TEPP inactive. This method (2) is possible only with irreversible inhibitors. The activity of all solutions incubated at various pH levels was then measured at pH 7.2 by the manometric technique. The results are given in Fig. 3.

One representative experiment may be described. An enzyme solution in 0.01 M phosphate buffer of pH 7.2 was used which hydrolyzed 10 gm. of acetylcholine per ml. per hour. Of this 0.2 ml. was diluted with 0.2 ml. of 0.1 M buffer of the desired pH and kept at 10° for about 30 minutes. 0.1 ml. of TEPP solution containing 0.2 γ per ml. in the same buffer was
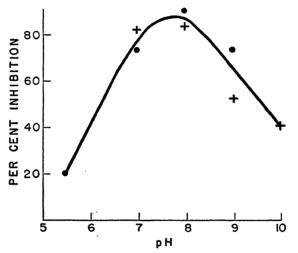
FIG. 3. Inhibition of acetylcholine esterase by TEPP as a function of pH. The concentrated enzyme was exposed to TEPP at various pH values, then rapidly diluted, and the activity tested at pH 7. The symbols represent two different sets of data.

added and the mixture kept for 15 minutes. The mixture was rapidly diluted 500 times with gelatin-bicarbonate buffer. In the control the TEPP solution was replaced by buffer.

It is noteworthy that the activity of the enzyme was not affected by exposure for a period of 1 hour to solutions with pH ranging from 5.5 to 10. The activity of the controls, when the pH was restored to 7.2, was equal in all cases. This indicates that the dependence of the enzyme activity on pH reflects reversible and not irreversible changes in the enzymatic activity within the experimental period.

Fig. 3 relates the inhibitory effect of TEPP to pH. In view of the extremely fast and irreversible reaction between enzyme and TEPP, the curve represents the true degree of reaction between the enzyme and the inhibitor.

<div align="center">

TABLE III

Inhibition of Acetylcholine Esterase by TEPP As Function of pH

</div>

The enzyme solution was diluted with buffer of the pH desired to give an activity of 53 mg. of acetylcholine split per ml. per hour. 0.4 ml. of this solution was used for each test-tube. TEPP was prepared in a buffer solution containing 0.024 γ per ml. Buffer was added to bring up the total volume to 4.1 ml. After 10 minutes incubation 3.6 mg. of acetylcholine in 0.9 ml. were added to each tube and readings taken 30 minutes later. 100 Klett units correspond to 1 μM of acetylcholine. The control without enzyme gave a reading of 450 Klett units. Addition of 1.2 ml. of TEPP did not change this value. Temperature 20°.

Estimated by graphic interpolation of these values, the amount of TEPP required for 50 per cent inhibition was 1.65 ml. for pH 7, 1.95 ml. for pH 8, and 2.4 ml. for pH 9. The C_{50} values were therefore 3.3, 4.1, and 4.9 × 10^{-8} M respectively.

• TEPP	pH 7		pH 8		pH 9	
	Klett units	Per cent inhibition	Klett units	Per cent inhibition	Klett units	Per cent inhibition
ml.						
0	246	0	254	0	187	0
0.6	208	15.4				
0.8	186	24.4				
1.0	172	30.1				
1.2	160	35.0	162	35.2		
1.4	152	38.2	161	36.6		
1.6	129	47.6	150	40.9	137	26.7
1.8			135	46.9	122	34.8
2.0			128	49.6	108	42.3
2.2			112	55.9	120	35.8
2.4			118	53.5	105	43.9
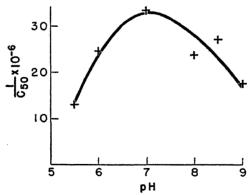
FIG. 4. Inhibitory action of TEPP as a function of pH. $1/C_{50}$ represents the reciprocal of the molar concentration of TEPP required for 50 per cent inhibition.

In another group of experiments, graded amounts of TEPP in the appropriate buffer were added to an enzyme solution diluted with the same

buffer and the mixture was incubated for 10 minutes. Acetylcholine was then added and the enzymatic hydrolysis determined colorimetrically after 10, 30, and 60 minutes. In this set of experiments the activity of the enzyme was measured at the same pH as that at which the reaction with TEPP took place. Therefore, it represents a summation of the effect of pH on the interaction of the enzyme with TEPP and with acetylcholine. One typical experiment is described in Table III. Fig. 4 summarizes the data obtained in this way. The activity of TEPP is expressed as $1/C_{50}$; i.e., the reciprocal of the concentration required for 50 per cent inhibition. Each of these figures represents an average of several experiments. The difference in the percentage inhibition of acetylcholine hydrolysis between pH 7 and 9 is relatively small and the values at the alkaline side appear to be less accurate than at the acid side.

DISCUSSION

The data permit certain conclusions as to the nature of the active surface of acetylcholine esterase. They suggest the existence of an electrically negative site (group of atoms) and a part at which the ester linkage is broken and which therefore may be called the "esteratic" site.

The esteratic site contains a group of atoms which behaves as a base. The conjugate acid is not active. There is also a group of atoms which behaves as an acid, the conjugate base of which is inactive. These two functions may involve two separate groups within the site, or the dual rôle may be played by the same group. This is represented by the following scheme.

$$EH_2^+ \xrightleftharpoons{+H^+} EH \xrightleftharpoons{+OH^-} E^- + H_2O$$

$$\text{Inactive} \qquad \text{Active enzyme} \qquad \text{Inactive}$$

These atomic groups may be part or the whole of the esteratic site.

This view is supported by the following facts. (a) Acetylcholine and TEPP do not undergo any changes with pH. Therefore, all changes of enzyme activity with pH must reflect changes in the protein structure. These changes permit the evaluation of the dissociation constants of the esteratic part, which will be reported in a subsequent paper. (b) The inhibitory action of TEPP shows an optimum between pH 7 and 8. Since TEPP is a neutral molecule, no attraction to the negative site is possible. The interaction with the protein must therefore occur at the esteratic site and the occurrence of a pH optimum of TEPP inhibition thus reflects changes of the esteratic site. Moreover, the experiment with prostigmine discussed below excludes any change in the negative site of the en-

zyme surface. The similar pH variation of acetylcholine hydrolysis substantiates these conclusions.

The existence of an electrically negative site, at least for solutions which are not very highly acid (pH higher than 5.5), may be reasonably assumed *a priori* in view of the positive electrical charge of its normal substrate, acetylcholine cation. The following experimental data support this view. (*a*) The cationic form of the closely related substrate dimethylaminoethyl acetate is hydrolyzed at a much greater rate than the free base. (*b*) The cationic form of eserine is a much more potent inhibitor of acetylcholine esterase than the base.

The rôle of the positive charge in the cationic forms is mainly electrostatic; that is, the positive charge and the changes in structure of the substrate which attend the conversion of base to acid are not involved in the forces which stabilize the critical complex except in so far as they increase the binding through electrostatic attraction. Stated in another manner, the reaction would be identical for both forms in a medium of infinite dielectric constant.

This view is supported by the following facts. (*a*) The dimethylaminoethyl acetate base is hydrolyzed, although it cannot be strongly attracted to the anionic center. (*b*) The base form of eserine is an inhibitor, although again no strong electrostatic attraction is possible. This shows that eserine and prostigmine must interact not only with the negative but also with the esteratic site. The protecting effect of the two compounds against DFP reported previously (2) must be interpreted in the same way.

If it is accepted that the only change in passing from the free base of dimethylaminoethyl acetate to the cationic form is to add the electrostatic attraction, it is possible to apply the Brønsted-Christiansen-Scatchard equation (6) and calculate the charge on the negative site and to estimate the distance of separation of the nitrogen and the negatively charged atom in the anionic center. From atomic radii this distance is estimated to be about 5 A.

The Brønsted-Christiansen-Scatchard equation relates the constant for a complex between ions to the dielectric constant and the ionic strength of the medium as follows:

$$\log K = \log K' - \frac{Z_A Z_B \epsilon^2}{2.3 D k T r} + \frac{Z_A Z_B \epsilon^2}{2.3 D k T} \times \frac{\kappa}{1 + r \kappa}$$

where K is the affinity constant, K' is the affinity constant for $D = \infty$, $\kappa = 0$, Z_A and Z_B are the number of electronic charges for ions A and B respectively, D is the dielectric constant, ϵ is the electronic charge, k is the Boltzmann constant, T the absolute temperature, $\kappa = \sqrt{(8\pi\epsilon^2/DkT)\mu}$, μ is the ionic strength, and r is the distance of closest approach.

Let Z_B apply to the enzyme and $Z_A = +1$ apply to dimethylaminoethyl acetate cation.   For the free base $Z_A = 0$.   Then

$$\log \frac{K \text{ (base)}}{K \text{ (cation)}} = \frac{Z_B \, \epsilon^2}{2.3 r D k T} \times \frac{1}{1 + r\kappa}$$

For $\mu = 0.2$, water at 25°, this equation reduces to

$$r = \frac{2.1 \times 10^{-10}}{\log \dfrac{K \text{ (base)}}{K \text{ (cation)}}} \times \frac{Z_B}{D}$$

where the value of D is to be estimated from the Schwarzenbach approximation (7).   The data for eserine and dimethylaminoethyl acetate indicate a 20-fold greater affinity constant for the charged reactant compared to the free base.   If $Z_B = -1$, $r = 6.5 \times 10^{-8}$ cm.   The assumption of $Z_B = -2$ would yield a larger value of r, namely 8.2 A, which is less satisfactory.   Thus the negative site bears a single electronic charge.   (The value for r is a little large, even for $Z_B = -1$.   But it must be remembered that there is possibly a positive grouping in the neighborhood which would weaken the negative field.   Also the data for pH 10 are subject to considerable experimental error, arising from the low values and from corrections for non-enzymatic hydrolysis.   All considered, including the high ionic strength, the value derived for r is satisfactory.)

Whether the negative site of the active surface plays its rôle exclusively through electrostatic forces or, in addition, is involved in the formation of the critical complex leading to the breaking of the ester linkage cannot be decided at present and requires further investigation.

The group of phosphates, which are active inhibitors, like TEPP, DFP, and parathion, differs from that of the similarly constituted but inactive phosphates in that during hydrolysis a bond involving a phosphorus atom is ruptured in an $S_N 2$ mechanism.   The enzyme attacks the phosphates in a nucleophilic substitution reaction, and therefore the part of the inhibitor entering the covalent bond with the enzyme has in all cases the structure $[(RO)_2 PO]^+$.   In the other phosphates a C—O bond is always broken during hydrolysis.

The proposed mechanism explains the decrease of inhibitory activity at low pH at which the esteratic site is occupied more and more by H ions.   However, the explanation for the decrease of inhibitory action at the alkaline side must await further investigation of the esteratic group.

SUMMARY

The inhibition of acetylcholine esterase by various inhibitors was investigated as a function of pH and related to the electric charge of the inhibi-

tor. These observations have revealed several features of the active surface of the enzyme.

1. Prostigmine cation inhibition is not affected by pH in the range tested (6 to 10).

2. Eserine inhibits strongly at pH 6. The inhibition decreases with increasing pH. Eserine cation is a stronger inhibitor than the base. However, at pH 10 the entire inhibition must be ascribed to the base, since the proportion of cationic form present at this pH was found to be negligible.

3. The rate of hydrolysis of dimethylaminoethyl acetate by acetylcholine esterase was tested at pH 6 to 10 and compared with the pH dependence of acetylcholine hydrolysis. The compound was found to exist in cationic and basic forms, depending upon the pH of the medium. The cation was found to be the more active form, but the neutral molecule was also hydrolyzed, though at a much lower rate.

4. TEPP inhibition shows a definite optimum between pH 7 and 8. This fact was demonstrated in two ways: (a) by measuring the inhibition at various pH values and (b) by exposing concentrated enzyme to the inhibitor at various pH values and determining at neutral pH the activity remaining after high dilution.

The data reveal that the active surface contains a group of atoms bearing a single negative charge. The active surface also contains an esteratic site which behaves in acid solution as a proton acceptor, in alkaline solution as a proton donor. Both the conjugate base and acid are inactive. The esteratic site is subject to electrophilic attack. This may explain the mechanism by which TEPP and similar phosphates, able to transfer phosphonium ions, combine with the enzyme.

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TRACER STUDIES OF GLUCURONIC ACID BIOSYNTHESIS*

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Glucuronic acid has been found in the urine of a variety of experimental animals (1, 2), chiefly in the form of conjugation products with phenolic substances and steroids (2, 3). The presence of glucuronides is widely interpreted as evidence of the ability of the animal organism to make use of the conjugation reaction to eliminate a great variety of toxic substances. Variations in the aglucone structures described in the literature make it evident that the enzymic control of conjugation is not highly specific.

The precursors of glucuronic acid or the mechanism of synthesis have not been established (2–11). The enzyme β-glucuronidase (12) apparently is not concerned with the synthesis of the glucuronic acid molecule, but acts merely as a conjugating enzyme (11). Although the structural similarity of glucose and glucuronic acid strongly suggests this carbohydrate as a precursor of glucuronic acid, many investigators (5–10) have concluded that glucose is not directly convertible to glucuronic acid. Glycogen (5, 6), lactic acid (7), the glycogenic amino acids (7), mucin (8), and certain 3-carbon intermediates of carbohydrate metabolism (9, 10) have been suggested as precursors.

In this investigation radioactive tracer techniques have shown that in borneol-treated guinea pigs the carbon atoms of glucose are converted to glucuronic acid without a change in the relative activity of carbon in position 6 compared with the total activity in positions 1 to 5. The slight conversion of radioactive sodium bicarbonate to glucuronic acid could be accounted for by an intermediate formation of glucose and glycogen. It was further shown that ascorbic acid is not converted to glucuronic acid in borneol-treated guinea pigs.

EXPERIMENTAL

Experimental Animals—Male guinea pigs of the Rockland Farms strain, 2 to 3 months old and weighing 300 to 450 gm., were used. Rockland

guinea pig diet and water were supplied *ad libitum* before and during the experiments. Before injection of the radioactive substances, *dl*-borneol (200 to 250 mg.) was administered by mouth as a homogenate in 2 to 2.5 ml. of evaporated milk. The quantities of bornyl glucuronide excreted in the urine during the experimental periods are listed in Table III.

Measurement of Radioactivity; Collection of Urine, Feces, Liver Glycogen, and Respiratory Carbon Dioxide—The techniques employed were the same as published previously (13), except that urines were collected under 2 ml. of toluene instead of oxalic acid.

Measurement of Glucuronic Acid—The method of Dische[1] (14) was found to be applicable to the urine of normal and borneol-treated guinea pigs. Recovery experiments were performed with added D-glucurone[2] (m.p. 175–177°; $[\alpha]_D^{27} = +18.9°$ in water), with an Evelyn photoelectric colorimeter. Quantitative recoveries were obtained within an estimated precision of ±10 per cent when the glucuronic acid content of the diluted solutions taken for analysis was less than 30 γ per ml. The glucuronic acid output of normal guinea pigs was approximately 28 mg. per day. This figure was subtracted from the glucuronic acid values obtained for borneol-fed animals in order to correct for the output of normal (unstimulated) glucuronides. A further correction was applied to allow for the 15 per cent reduction in color intensity of glucuronic acid when conjugated through the glucosidic bond of the glucuronide. This correction was found to be necessary both for bornyl glucuronide and for zinc bornyl glucuronidate.

Isolation of Zinc Bornyl Glucuronidate Dihydrate (ZBG)—Glucuronic acid was isolated from the urine of borneol-treated guinea pigs as the zinc salt of bornyl glucuronide (15). A 24 hour urine sample was diluted to about 50 ml. with water and centrifuged, the precipitate washed once with 5 ml. of water, and the washings added to the diluted urine. The precipitate was discarded. Glacial acetic acid was then added to obtain a pH of 4. Carrier bornyl glucuronide[3] (200 to 400 mg.) was added and the solution was shaken thoroughly. The acidified urine was treated with saturated lead acetate solution until precipitation was complete. Excess lead ion was removed and the pH adjusted to 1.0 by addition of 1 M sulfuric acid. The solution was filtered with Celite filter aid, diluted to 100 ml. with water, and extracted with ethyl ether in a continuous extractor for 24 hours. The ether extract was decolorized with charcoal and the

[1] We are indebted to Dr. Z. Dische for his advice in applying the analytical procedure.

[2] Generously supplied by the Corn Products Sales Company, New York 4.

[3] Obtained from Rocky Mountain Research, Inc., Denver, Colorado.

ether evaporated under a water pump vacuum, leaving a concentrated aqueous phase. The solution was filtered, heated to boiling, and stirred with 500 mg. of zinc acetate dihydrate. The precipitate of ZBG was washed with boiling water, absolute ethanol, and ethyl ether and dried for 24 hours *in vacuo* over calcium sulfate. With carrier-free material, the chemical identity of the isolated ZBG was established by elementary analysis, optical rotation,[4] determination of the neutralization equivalent, and the Dische colorimetric method.

$$C_{32}H_{50}O_{14}Zn \cdot 2H_2O.$$ Calculated. C 50.59, H 7.11, Zn 8.56
                                Found.   " 50.39, " 6.78, " 8.69

The following experiments were performed to establish the radioactive purity of the ZBG isolated after the administration of radioactive glucose and bicarbonate.

TABLE I

Specific Activity of Zinc Bornyl Glucuronidate Isolated from Guinea Pig Urine

Recrystallizations	Animal G-2	Animal G-5
	Glucose fed†	NaHCO₃ fed†
None	294	28.6
1	324	25.6
2	320	29.4
3	333	29.0

* Details of these experiments are listed in Table III.
† Specific activity in counts per minute per mg. of ZBG.

The specific activity of the recrystallized ZBG remained constant over the course of two additional recrystallizations from 1.8 M sulfuric acid. The recrystallizations were carried out by dissolving the ZBG in 1 to 3 ml. of 1.8 M sulfuric acid, diluting to 10 ml. with water, adjusting to pH 5 by the addition of anhydrous sodium acetate, and reprecipitating the ZBG from the boiling solution by the addition of 500 mg. of zinc acetate dihydrate. The precipitate of ZBG was then washed and dried as described above. Typical recrystallization data are listed in Table I.

It was further shown that the relatively high specific activity of the ZBG isolated following the injection of radioactive glucose was not due to contamination of the ZBG with small amounts of highly radioactive glucose or other metabolic products. In one experiment ZBG was isolated from the urine of a guinea pig which had not been injected with radioactive glucose, but radioglucose (0.488 mg., 2.77×10^5 c.p.m.) had been

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[4] $[\alpha]_D^{20} = -49.5°$ (0.5 per cent solution in 1.8 M sulfuric acid).

added to the collection flask at the start of the experiment. When ZBG was isolated from the urine in the usual manner, it was found to be non-radioactive within the sensitivity of the method. In another experiment radioactive glucose (20.1 mg., 1.139×10^7 c.p.m.) was injected into a guinea pig which had received no borneol. Carrier bornyl glucuronide was added to the urine and isolated as ZBG. It was found to be non-radioactive within the sensitivity of the method.

The barium salt of the p-bromophenylosazone of glucuronic acid (16), prepared from the ZBG in the experiment on Animal G-8 (Table III), had the same radioactivity per mole as the ZBG from which it was prepared. The barium salt was prepared by hydrolysis of the ZBG for 3 hours with 0.05 N sulfuric acid, neutralization of the hydrolysate with saturated barium hydroxide, and formation of the osazone. The melting point of the derivative is reported as 215–217°. The barium salt obtained from the ZBG melted at 213–215° with decomposition. (A sample prepared from pure glucurone had a melting point of 216–218° with decomposition. The mixed melting point of these samples was 213–216° with decomposition. It should be noted that the reported molecular formula for this substance (16) is in error.)

Preparation of Labeled Substances—C^{14}-glucose, labeled uniformly in all positions, was prepared photosynthetically (17).[5] Its specific activity (as barium carbonate) was 5.67×10^5 c.p.m. per mg. $NaHC^{14}O_3$ was obtained[6] in an aqueous solution containing 1 mM of $NaHCO_3$ in 5 ml. of solution. The radioactivity of this solution (as barium carbonate) was 1.31×10^8 c.p.m. per ml. Ascorbic acid labeled with C^{14} in the carboxyl group was prepared by the addition of C^{14}-cyanide to L-xylosone (22).[5] Its specific activity (as barium carbonate) was 8.05×10^5 c.p.m. per mg.

Decarboxylation of ZBG—Quantitative decarboxylation of L-ascorbic acid has been carried out in 20 per cent sulfuric acid at 100° in an atmosphere of nitrogen and with heating periods of 3 to 4 hours (18). Similar conditions were used for the quantitative hydrolysis-decarboxylation of ZBG, except that longer heating periods (16 to 24 hours) were necessary. The carbon dioxide was collected in saturated barium hydroxide solution and counted as barium carbonate. Within the precision of the method (± 3 per cent) the yield of barium carbonate from the carboxyl group of glucuronic acid was quantitative.

RESULTS AND DISCUSSION

Table II contains data showing the rate of excretion of radioactive carbon dioxide in the expired air of borneol-treated guinea pigs. These

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animals had received radioactive glucose and radioactive sodium bicarbonate by intraperitoneal injection. The rate of oxidation of glucose by borneol-treated guinea pigs was similar to the rate reported by Zilver-

TABLE II

Respiratory C^{14}-Carbon Dioxide of Borneol-Treated Guinea Pigs Following Injection of C^{14}-Glucose and C^{14}-Sodium Bicarbonate

Substance injected	Animal No.	Weight	Per cent of administered dose expired as CO_2 after										
			1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	10 hrs.	24 hrs.	25 hrs.
		gm.											
$NaHC^{14}O_3$*	G-5	325	72.9	85.5	89.6	90.9	91.7		92.5			93.9	
	G-9	390	58.6	77.9	84.3	86.2	87.4		88.4			90.6	
C^{14}-Glucose†	G-2	330	6.6	25.8	41.0	48.9	54.1	57.3		61.7	64.7	70.5	70.8
	G-8	407	5.3	20.8	32.2	40.7	46.7	50.0		56.3	59.4	66.2	66.5

* Animals G-5 and G-9 received 1.68 mg. of radioactive sodium bicarbonate, 1.31 $\times$ 10^7 and 1.25 $\times$ 10^7 c.p.m. per mg., respectively.

† Animals G-2 and G-8 received 20.1 mg. of radioactive glucose, 1.139 $\times$ 10^7 c.p.m. per mg.

TABLE III

Distribution of Radioactivity after Intraperitoneal Injection of C^{14}-Sodium Bicarbonate, C^{14}-Glucose, and 1-C^{14}-L-Ascorbic Acid

Substance injected	Animal No.	Weight	Borneol fed	Urinary bornyl glucuronide	Per cent of injected dose found in				
					Glucuronic acid	Expired air	Urine	Feces	Liver glycogen
		gm.	*mg.*	*mg.*					
$NaHC^{14}O_3$*	G-5	325	200	437	0.18	93.9	1.41	0.62	0.0
	G-9	390	250	550	0.29	90.6	1.50		
C^{14}-Glucose†	G-2	330	200	282	1.79	70.8	4.48	1.09	0.19
	G-8	407	250	452	4.05	66.5	5.37		
1-C^{14}-L-Ascorbic acid‡	G-11§	448	350	212	0.0		6.84		

* 24 hour experiment; dosage indicated in Table II.

† 25 hour experiment; dosage indicated in Table II.

‡ The animal received by intraperitoneal injection 3.83 mg. of 1-C^{14}-labeled L-ascorbic acid (3.08 $\times$ 10^6 c.p.m. per mg.) (22).

§ This animal had received no ascorbic acid in its diet for 4 days prior to the injection of radioactive ascorbic acid; 24 hour experiment.

smit *et al.* (19) for normal rats. Similarly, the record of respiratory carbon dioxide after bicarbonate injection resembled that obtained by other investigators (20, 21) using rats.

Table III contains data showing the distribution of radiocarbon in urinary glucuronic acid, expired air, urine, feces, and liver glycogen from

borneol-treated guinea pigs injected with radioactive glucose, sodium bicarbonate, and 1-C^{14}-L-ascorbic acid. The data in Table III show that the carbon atoms of both glucose and carbon dioxide (as sodium bicarbonate) can be utilized by the borneol-stimulated guinea pig for glucuronide synthesis. Ascorbic acid (or at least its carboxyl carbon) is not available as a precursor of glucuronic acid in sufficient quantity to provide a positive test. This latter result is not unexpected, because it had been reported earlier (23, 24) that scorbutic guinea pigs can synthesize glucuronic acid. The possibility is not excluded, however, that the mechanism of glucuronide formation may differ, depending on the need of the animal for glucuronic acid for detoxication or synthesis of tissue uronide. Studies on this aspect of glucuronic acid formation are under way.

The incorporation of radiocarbon into urinary glucuronic acid after the administration of radioactive glucose suggests that the reactions transforming glucose into glucuronic acid are relatively direct and do not involve an appreciable biological dilution. To obtain further information on this point the radioactive ZBG isolated from the urine of several animals was decarboxylated. If the glucose molecule is transformed into glucuronic acid without breaking the carbon chain, the carboxyl group of the ZBG obtained following administration of radioactive glucose should contain one-sixth of the total activity of the ZBG. On the other hand, the ZBG from animals injected with radioactive sodium bicarbonate should contain very little radioactivity in the carboxyl groups if the radioactivity of the glucuronic acid is derived from glucose which in turn had acquired its radioactivity by carbon dioxide fixation (25).

The results of the decarboxylation experiments are summarized in Table IV. The data show that, after the administration of radioactive glucose, one-sixth of the total activity of the glucuronic acid was found in the carboxyl group. Thus it appears that the glucose carbon chain is not broken before being converted to glucuronic acid, or, if so, the respective fragments were recombined without a detectable dilution effect. Although this result argues against a bornyl trioside precursor of glucuronic acid (9, 10), further degradation studies with unsymmetrically labeled glucose (now being carried out in this laboratory) are needed.

After injection of radioactive bicarbonate, only a small fraction of the total ZBG activity is found in the carboxyl group, 4.6 per cent at the end of 24 hours and only 1.8 per cent at the end of 7.5 hours. Since injection of labeled bicarbonate gives rise to 3,4-labeled liver glycogen (25), it is likely that the same distribution of radioactivity would be true for glucuronic acid obtained after injection of sodium bicarbonate. The small activity found in the carboxyl group is probably derived from small

amounts of labeled intermediates arising from side reactions of glycolysis, as in the dephosphorylation of dihydroxyacetone (26).

Although the glucose and liver glycogen of the organism are in a state of dynamic equilibrium, the high specific activity of the glucuronic acid obtained after administration of radioglucose suggests that the glucose does not necessarily have to enter the glycogen store in order to be converted to glucuronic acid.

TABLE IV

Distribution of C^{14} in Carboxyl Groups of Zinc Bornyl Glucuronidate Isolated from Urine of Borneol-Treated Guinea Pigs Following Injection of C^{14}-Glucose and C^{14}-Sodium Bicarbonate

Substance injected	Animal No.*	Weight of ZBG	Total radio-activity†	Carboxyl activity†	Theory†‡	Difference§
		mg.				
C^{14}-Glucose	G-2	20.45	6,827	1102	1138	−3.3
	G-2	13.20	4,396	724	733	−1.1
	G-8	32.70	14,010	2330	2335	−0.2
	G-8	29.60	12,680	2260	2114	+6.8
NaHC^{14}O$_3$	G-9	67.95	2,072	100.0	345	
	G-9	64.75	1,946	84.3	324	
	G-10*	39.60	5,084	84.7	848	
	G-10	57.45	7,383	133.8	1231	

* The numbers refer to the animals of Table III, except that Animal G-10 represents an experiment of only 7.5 hours duration. Dosage, 6.45 × 10⁷ c.p.m. per mg.

† Activity in counts per minute per mg.

‡ This column shows the expected radioactivity of the carboxyl groups as one-sixth of the total ZBG radioactivity.

§ Per cent difference between expected and found carboxyl radioactivity.

SUMMARY

1. Glucuronic acid synthesis by borneol-treated guinea pigs was investigated by radioactive tracer techniques.

2. Of the administered glucose activity, 1.79 to 4.05 per cent was recovered in urinary bornyl glucuronide within 24 hours. After a similar interval only 0.18 to 0.29 per cent of administered C^{14}-sodium bicarbonate activity was found in urinary bornyl glucuronide, and no radioactivity was detectable in the glucuronic acid following an injection of 1-C^{14}-L-ascorbic acid.

3. Partial degradation of the isolated glucuronic acid indicated that it contained the same ratio of C^{14} in position 6, compared to total activity, as that observed in the initial glucose. Hence the carbon chain of glucose

is used directly for glucuronide synthesis, or the sequence of steps does not permit a dilution effect in other positions during fragmentation.

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MECHANISM OF INHIBITION OF ANAEROBIC GLYCOLYSIS OF BRAIN BY SODIUM IONS*

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During the course of an investigation of the anaerobic glycolysis of nervous tissue of cotton-rats, it was observed that sodium ions had a considerable inhibitory effect under certain conditions. These results are in accord with an earlier report by Racker and Krimsky (1) who found that Na^+ inhibited the glycolysis of mouse brain homogenates. LePage (2) has reported that the glycolysis of tumor homogenates is much reduced when sodium salts of buffers and substrates are employed as compared with glycolysis in the presence of potassium salts. On the basis of rather general observations, Na^+ has been excluded from systems with homogenates of liver (3, 4) and chick embryo (5) for the study of glycolytic and oxidative reactions of carbohydrate metabolism. In view of the lack of specific information concerning the nature of the inhibition caused by Na^+, an attempt has been made to investigate the extent, specificity, and mechanism of the inhibition on preparations obtained from nervous tissues. The results are presented in this communication.

The report of Racker and Krimsky was one of the first to emphasize the inhibitory nature of Na^+ upon metabolic processes, although the phenomenon might have been predicted from earlier studies by various workers on the stimulatory effect of K^+. In the most definitive studies of the stimulatory effect of K^+ upon carbohydrate metabolism, Boyer et al. (6, 7) concluded that K^+ stimulated the transfer of phosphate from phosphopyruvic acid to adenylic acid.[1] These workers also mentioned that a high concentration of Na^+ was inhibitory to the same reaction. There have been numerous other studies showing the stimulatory effect of K^+ upon glycogen deposition by liver slices (8), fermentation by yeast extracts (9, 10), and aerobic glycolysis of brain (11). In some of these studies, it seems likely that the effect under study could have been termed an Na^+ inhibition as well as a K^+ stimulation if the experiments had been performed in a different fashion.

[1] The following abbreviations have been used: HDP, hexose diphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenylic acid; Apyrase, adenylpyrophosphatase; FMP, fructose monophosphate; DPN, diphosphopyridine nucleotide; ATPase, adenosinetriphosphatase.

Materials and Methods

Sodium and potassium salts of hexose diphosphate were prepared from the commercial barium salt. The solutions contained a small amount of an unknown inhibitor which was removed by treating briefly with charcoal.

Sodium and potassium phosphopyruvate were prepared from the silver barium salt of a sample synthesized by the method of Schmidt.[2]

ATP was isolated from rabbit muscle by a method closely resembling that recently described by Dounce *et al.* (12).

ADP was prepared from ATP by an unpublished method which utilized an ATPase obtained by isoelectric precipitation at pH 6 of a water extract of rabbit muscle, as described by Kalckar (13) for the hydrolysis of the terminal phosphate. The ADP was isolated as the barium salt after purification as the mercury salt. Enzymatic analyses with hexokinase and AMP deaminase showed an ATP and AMP content, respectively, of less than 2 per cent for each component.

AMP was prepared by neutralization of a sample of the commercial acid.[3] DPN was prepared by the method of Williamson and Green (14). The various preparations used in these experiments had a purity of 40 to 60 per cent.

Cotton-rats weighing 90 to 150 gm., obtained from Tumblebrook Farms, were used in this study. The animals were decapitated while under light ether anesthesia and the brain and cord removed. The medulla was detached and pooled with the cord. The remaining brain tissue was homogenized thoroughly with 10 volumes of ice-cold 1.6×10^{-4} M ammonium phosphate buffer (pH 7.4) in a Potter-Elvehjem homogenizer. The cord-medulla mixture was homogenized with 8 volumes of cold water. Extracts were prepared from the homogenates by centrifuging for 15 minutes at 1800 r.p.m. in a Servall angle centrifuge and carefully removing the supernatant.

The manometric experiments were carried out at 38° in Warburg vessels with a total volume of 7 to 9 ml. by the following experimental procedure. The main chamber of the vessel contained the following substances, with molarity expressed on the basis of the final volume of reactants: 1.4×10^{-2} M glucose, 1×10^{-3} M ATP, 4×10^{-3} M $MgCl_2$, 2.4 or 4.8×10^{-2} M $KHCO^3$, 4.7×10^{-4} M reduced glutathione, 5×10^{-4} M DPN, and 1.3×10^{-3} M nicotinamide. In some experiments sodium or potassium HDP was added to give a final concentration of about 2.5×10^{-3} M; in others $NaHCO_3$ has been substituted for $KHCO_3$ and in many experiments NaCl or KCl has

been added. The side arm of the vessel contained 0.05 ml. of 0.08 M KHCO$_3$, 0.05 ml. of 0.08 M ammonium phosphate buffer (pH 7.4), and 0.15 ml. of a 1:10 homogenate of the tissue. The total volume of the cup contents was 1.0 to 1.2 ml. The tissue was added as the last step in the procedure before the vessels were gassed at room temperature for 7 minutes with a mixture of 95 per cent N$_2$-5 per cent CO$_2$, and the contents of the vessel were mixed with the tissue as the vessel was placed on the bath.[4] After 7 to 9 minutes equilibration, the first reading was taken. In some cases, the gas evolution for the first 15 minute period following equilibration was not a reliable indication of lactic acid production, since hydrolysis of ATP and other factors may give anomalous gas changes during this period. The 15 to 60 minute period gave a fairly constant rate of acid production in most experiments and has been chosen as representative of the glycolytic rate. In some cases chemical analyses were made upon the contents of the vessel at the end of 60 or 90 minutes. In these experiments, the manometric values were extrapolated to coincide with the incubation time in order that the chemical and manometric values might be compared. All manometric experiments were carried out in duplicate or triplicate.

In the measurement of individual enzymes of the glycolytic system it has been convenient to carry out the reactions in Thunberg tubes. The conditions closely followed those used in the manometric studies with the exception of certain substrates or coenzymes which were added or omitted according to the reaction under study. The homogenate was placed in the side arm with the protecting buffers as described above and the Thunberg tubes filled with 5 per cent CO$_2$-95 per cent N$_2$ by three cycles of evacuation and filling. The reaction was stopped at the indicated time by the addition of the appropriate deproteinizing agent.

Analyses of inorganic phosphate were run on trichloroacetic acid filtrates by a modification of the Fiske and Subbarow method (16). In case preliminary hydrolysis of organic phosphate compounds was necessary, the methods are described in conjunction with the experiments.

Glucose and lactic acid were determined on aliquots of a Somogyi filtrate (17) by the methods of Nelson (18) and Barker and Summerson (19) respectively.

[4] In order to obtain full activity it was found necessary to observe carefully the precautions of buffering the tissue in the side arm, gassing at room temperature, and mixing the tissue with the other components at the time the vessel was placed on the bath. When the common procedure of gassing vessels at 38°, with shaking, with the tissue unprotected by buffers is followed, most of the glycolytic activity of brain homogenates is lost. It has been shown previously (15) that phosphohexokinase is inactivated by the slight acidification produced by such a procedure.

Results

The effect of sodium and potassium ions upon the production of acid from glucose by brain homogenates is shown in Fig. 1. It will be noted that a high rate of glycolysis obtains when all additions were made as potassium and ammonium salts. The addition of 0.07 M NaCl to this reaction mixture caused a striking reduction in the rate, as shown by the middle curve, although the activity is higher than that of an experiment in which only sodium salts were used. The effect of the same series of ion combinations on glycolysis of a glucose-HDP substrate is also shown in Fig. 1. In the presence of HDP, the addition of 0.07 M NaCl to a K^+-NH_4^+ medium has little inhibitory effect. Even with all sodium salts, a reasonable rate
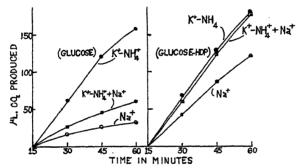
FIG. 1. Inhibition of brain glycolysis by NaCl and its reversal by HDP. In the experiments represented by the right-hand half of the graph 2.5 × 10⁻³ M potassium or sodium HDP was added. Other experimental conditions as described in the text.

of glycolysis is obtained in the presence of HDP, demonstrating the reversal of the Na^+ effect by the ester.

These experiments are in general agreement with the results of Racker and Krimsky (1), although the experimental procedure, as well as the species of experimental animal, was different in the two series of experiments.

Balance Studies on Sodium Inhibition—In the presence of HDP, the glycolytic activity of brain homogenates, as measured by acid production, is essentially unaffected by the addition of Na^+ to a medium which already contains potassium and ammonium salts, as shown in Fig. 1. When a chemical balance of the glycolytic process is carried out, however, Na^+ causes certain alterations in the reactions, even though the over-all activity is not changed. Such an experiment is presented in Table I. Also, extension of the Na^+ inhibition to cord-medulla preparations is demonstrated in this experiment.

In Table I, in addition to the total acidity changes as measured by CO_2 production, lactic acid, glucose, and inorganic phosphate were determined chemically. Measurements of the latter two substances give a picture of the intermediate phosphorylation reactions. In the presence of a sufficient phosphate reservoir such as HDP, some of the phosphorylation reactions may be inhibited substantially without slowing the over-all production of lactic acid, but the change in the rate of the phosphorylation reactions will be shown by a decreased esterification of inorganic phosphate and usually by an accompanying decrease in glucose utilization.

TABLE I

Chemical Analysis of Effect of NaCl on Glycolysis of Glucose-HDP by Homogenates and Extracts of Nervous Tissue

Results expressed as Q values (microliters per mg. of dry weight per hour). In these experiments, the components of the reaction mixture were those described under "Methods" with the following changes. In the Na^+ experiments, 0.024 M $NaHCO_3$ was present, but K^+ salts of ATP and HDP gave a K^+ concentration of about 1×10^{-2} M and ammonium phosphate buffer gave a final ammonium concentration of 8×10^{-3} M. In the K^+ experiments 0.024 M $KHCO_3$ replaced the Na^+ buffer. The vessels contained homogenate or extract equivalent to 2 mg., dry weight, of the original tissue. The experiments with extracts were conducted for 90 minutes; with homogenates for 60 minutes.

Measurement	Buffers	Brain homogenate	Brain extract	Cord homogenate	Cord extract
CO_2 produced	Na^+	62.4	40.3	29.2	26.6
	K^+-NH_4^+	66.4	44.8	29.3	27.4
Lactic acid produced	Na^+	60.7	38.3	28.7	24.3
	K^+-NH_4^+	61.7	43.6	31.6	29.1
Glucose utilized	Na^+	23.8	22.0	9.6	11.8
	K^+-NH_4^+	37.8	28.5	16.0	16.9
Inorganic phosphate utilized	Na^+	−0.5	24.5	−3.3	10.1
	K^+-NH_4^+	23.4	32.3	11.6	15.8

For sake of comparison the values are expressed as Q values. In general, in accord with the results of Fig. 1, Na^+ caused no appreciable decrease in lactic acid production or total acid production in any of the four different preparations tested. It is also apparent that the chemical and manometric values are in quite good agreement in the various cases. In contrast to the acid values, the utilization of glucose was inhibited by the addition of NaCl in all four cases, although the effect was somewhat smaller with the extracts than with the corresponding homogenates. In the case of the two homogenates, the glucose utilization was not large enough to account for the lactic acid production. For example, with the brain homogenate, the glucose utilized amounted to 23.8 μl. per mg., which could account for only 47.6 μl.,

of lactic acid, while the actual production was 60.2 μl. The balance of the lactic acid was formed from the HDP, of course.

The inorganic phosphate changes follow the same pattern as the glucose values, although the effect of Na⁺ is perhaps even more striking. With the homogenates no net esterification of inorganic phosphate occurs in the presence of Na⁺, although considerable net esterification occurs with only K⁺ and NH₄⁺ present. It is interesting to note that the changes caused by the Na⁺ are again much smaller with the extracts. It is possible to suggest two hypotheses from the foregoing observations: (a) that Na⁺ acts in some fashion to decrease the phosphorylative efficiency of the glycolytic system, thereby creating an organic phosphate deficit which must be rectified by the addition of an organic phosphate donor such as HDP; (b) that, since the effect is considerably reduced in extracts as compared with homogenates, one or more of the enzymes affected by Na⁺ is removed by centrifugation.

Because of the inhibition of esterification processes, it seemed likely that Na⁺ is affecting one or more of the following reactions: (1) hexokinase, (2) phosphohexokinase, (3) the phosphorylation coupled with the oxidation of glyceraldehyde phosphate, (4) the transfer of phosphate from phosphopyruvate to the adenylic acid system, and (5) Apyrase.

An inhibition by Na⁺ of one or more of the first four reactions would decrease the glycolytic rate by lowering the rate of ATP formation or utilization. A stimulatory effect upon the fifth enzyme, Apyrase, would produce the same effect by lowering the concentration of ATP.

In a later section the effect of Na⁺ upon each of these reactions is described. Before proceeding to the testing of the individual enzymes, we attempted to obtain more information concerning the inhibition of the entire glycolytic system, particularly in so far as concentration effects and specificity were concerned.

Concentration of Sodium Ions—In Fig. 2 various concentrations of NaCl were superimposed upon the usual K⁺-NH₄⁺ buffer system which was described earlier. The results varied somewhat from preparation to preparation, but the experiments are typical. NaCl was markedly inhibitory at concentrations as low as 0.035 M in most experiments and the inhibition increased rapidly with increasing NaCl concentration.

In no experiment did we detect appreciable inhibition with concentrations of NaCl below 0.01 M. From a practical standpoint, this observation demonstrates that it is not necessary to exclude rigidly all Na⁺ from the reaction mixture, but merely to hold the concentration at a low level.

The converse experiment is shown in Curve B of Fig. 2. Here a glycolytic system containing all Na⁺ was employed and KCl was added in increasing amounts. Added KCl at the lowest level tested, 0.014 M, more

than doubled the activity of the control, but it was impossible to increase the activity further by adding more KCl, and high concentrations actually proved inhibitory. From the results of this experiment it would appear that, although the Na^+-K^+ effect is reciprocal in nature, it is not probable that the effect upon glycolysis can be expressed as a simple ratio of the two

FIG. 2

FIG. 3
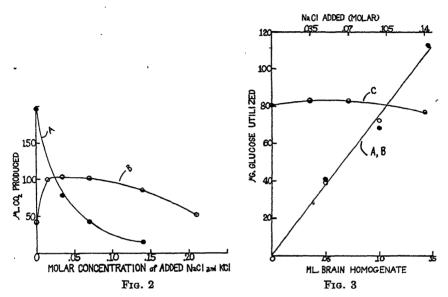
FIG. 2. Effect of NaCl and KCl concentration on glycolysis of brain homogenates. The experimental conditions were those described in the text with a K^+-NH_4^+ medium to which was added NaCl (Curve A) or KCl (Curve B) added to an Na^+ medium.

FIG. 3. Effect of NaCl on the hexokinase activity of brain homogenates. The experiments were conducted in Thunberg tubes at 38° for 4 minutes under 5 per cent CO_2-95 per cent N_2. The tubes contained 2×10^{-3} M ATP, 1.4×10^{-3} M glucose, 2×10^{-2} M KF, 4×10^{-3} M $MgCl_2$, and K^+-NH_4^+ buffers in a total volume of 1.0 ml. In Curve A ($\bullet$), 0.07 M NaCl was added, while in Curve B (O), no Na^+ was present. In Curve C the indicated amounts of NaCl were added. 0.1 ml. of brain homogenate was used in the experiments of Curve C.

ions. If this were so, the activity in Curve B should have increased proportionately to the KCl concentration.

Specificity of NaCl in Inhibition of Glycolysis—In view of the inhibition of higher concentrations of KCl it was desirable to learn whether the NaCl effect was a non-specific one. In Table II the effect of the addition of various concentrations of other salts to the usual K^+-NH_4^+ salt-glycolytic system is presented. The results are reported in terms of per cent inhibition of glycolysis. In contrast to the significant inhibition by Na^+ at a

level of 0.035 M, a similar concentration of KCl actually appeared to be stimulatory and a significant inhibition appeared only at a concentration of 0.14 M. LiCl resembled KCl rather than NaCl in its effect. The fact that KCl and LiCl were not inhibitory except in high concentration demonstrates that the inhibition by lower concentrations of NaCl is due neither to an increased tonicity of the reaction medium nor to the chloride ions.

In other experiments not shown here, calcium ions have been found to be inhibitory in concentrations as low as 0.02 M and sulfate ions caused marked inhibition in concentrations as low as 0.04 M. The mechanisms of the inhibition of glycolysis by calcium and sulfate ions have not been investigated further.

Effect of Sodium Ions on Hexokinase—Racker and Krimsky (1) suggested that the inhibition of brain glycolysis by Na+ was exerted on the hexokinase

TABLE II

Specificity of Inhibition of Glycolysis by NaCl

In this experiment, the glycolytic medium contains the K+-NH₄+ salt mixture previously described, to which were added the various concentrations of the salts as indicated. The manometric readings for the 15 to 60 minute period were used for the calculations.

Concentration of added salt	Inhibition produced by		
	NaCl	KCl	LiCl
M	per cent	per cent	per cent
0.035	47.3	−16.4	
0.07	78.0	3.3	5.6
0.14	85.2	48.9	53.8

reaction, since they observed that the production of lactic acid was influenced far less by Na+ with HDP and FMP than it was with glucose. Recently Wiebelhaus and Lardy (20) reported that Na+ in high concentrations was inhibitory to partially purified hexokinase obtained from beef brain.

The effect of NaCl upon the hexokinase reaction in brain homogenate of the cotton-rat is shown in Fig. 3. The reaction was followed by chemical determination of glucose disappearance in a filtrate free from the various phosphate esters which interfere with glucose determinations. Since the hexokinase reaction is essentially irreversible, it is not appreciably influenced by subsequent reactions as long as an adequate supply of ATP is available. Other methods of hexokinase assay, such as the manometric method of Colowick and Kalckar (21) in which the activity is followed by acid production, were found inapplicable to homogenates in which phos-

phatase activity and other phosphate transfer reactions may influence the acid production also. Likewise, with a complicated system, the disappearance of ATP may be influenced by reactions other than hexokinase. Glucose disappearance, appears, however, to be equivalent to hexokinase activity, even in complicated systems.

The effect of 0.07 M NaCl upon the hexokinase reaction in brain homogenates of the cotton-rat at different levels of tissue is shown in Fig. 3 in Curves A and B, where glucose disappearance has been plotted against the concentration of tissue. The relationship is linear, indicating that the assay is valid and that ATP is not a limiting factor. It will be noted that the NaCl had no appreciable effect despite the fact that similar concentrations of NaCl cause a 75 per cent inhibition of the glycolysis. This experiment, in which each concentration of brain was run in triplicate, is entirely representative of experiments with some half dozen different brain homogenates. The conditions were essentially those of the glycolytic experiment with the exception of the shorter incubation period. This alteration was necessary because it was not feasible to maintain the level of ATP required for maximum activity over longer periods of time.

The effect of varying concentrations of NaCl upon the same reaction is plotted in Curve C of Fig. 3. This experiment also shows that the NaCl had no influence with the exception of the highest concentration, 0.14 M, at which a slight inhibition was observed.

The foregoing results do not support the hypothesis that the inhibition of glycolysis by Na^+ operates through an effect upon hexokinase. The observation that Na^+ inhibits the fermentation of glucose but not of HDP and FMP could be explained by an inhibition of hexokinase, but an alternative explanation may be offered. The molar requirements of phosphate (as ATP) required to initiate fermentation on glucose, fructose-6-phosphate, and HDP are 2:1:0 respectively. Therefore, an inhibition of any reaction influencing the formation or destruction of ATP will be noted much more readily on a glucose substrate, since the ATP requirement is higher.

Although our results are in apparent disagreement with the inhibition of hexokinase observed by Wiebelhaus and Lardy, it should be noted that the species from which the brain was obtained was different. Also the latter authors were using a partially purified hexokinase rather than a homogenate, and the absence of other proteins may have influenced the properties of the enzyme. The inhibition found by Wiebelhaus and Lardy, 18.5 per cent at a level of 0.06 M NaCl and 37.2 per cent at 0.1 M NaCl, is considerably smaller than that observed on the entire glycolytic system.

The experimental period used in these experiments was short, but in experiments on the phosphorylation of glucose by hexokinase coupled with the oxidation of phosphoglyceraldehyde, as described in the next section,

an experimental period of 30 minutes was employed and no appreciable inhibition by Na$^+$ was observed.

Effect of Na$^+$ on Phosphohexokinase and Phosphorylation Coupled with Oxidation of Phosphoglyceraldehyde—The effect of 0.07 M NaCl upon the second phosphorylative step of glycolysis was also investigated. The phosphohexokinase activity of cotton-rat brain homogenates was measured in Thunberg tubes after incubation with FMP, ATP, MgCl$_2$, and the usual buffers together with KF to prevent Apyrase activity and potassium iodoacetate to prevent further oxidation of phosphoglyceraldehyde. The endproducts of the reaction were HDP and dihydroxyacetone phosphate and phosphoglyceraldehyde in an equilibrium mixture. After determination of the triose phosphates by alkaline hydrolysis (22) the HDP could be calculated from the equilibrium constant.[5] The sum of HDP-P and triose phosphate P divided by 2 gives the phosphate transferred from ATP to FMP and can be used as a measure of phosphohexokinase activity.

The results of a typical experiment are shown in Table III. The addition of the Na$^+$ seemed to cause a slight increase in the rate of transfer but this difference is neither constant nor significant. The Q value is given to show that the reaction rate is reasonably proportional to enzyme concentration and is also rapid enough to be consistent with the over-all glycolytic rate. It should be noted that the conversion of 1 molecule of FMP to HDP will be equivalent to the production of 2 molecules of lactic acid at a later stage in the reaction.

The third phosphorylation of the glycolytic system is that coupled with the oxidation of phosphoglyceraldehyde. These reactions were measured by incubating brain homogenates with HDP, ATP, pyruvate, glucose, MgCl$_2$, KF, reduced glutathione, and the usual buffers. After 30 minutes, the lactic acid production and the inorganic phosphate uptake were determined. The lactate production gives information concerning the oxidative reaction (Reaction 2).

(1)   HDP $\rightleftarrows$ glyceraldehyde phosphate + dihydroxyacetone phosphate

(2) Glyceraldehyde phosphate + inorganic phosphate + pyruvate $\xrightleftharpoons{\text{DPN}}$ 1,3-diphosphoglycerate + lactate

(3)   1,3 Diphosphoglycerate + ADP $\rightleftarrows$ 3-phosphoglycerate + ATP

(4)   Glucose + ATP $\xrightarrow{\text{hexokinase}}$ glucose-6-phosphate + ADP

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[5] The value for the equilibrium constant of zymohexase, 7.3×10^{-3} at 40°, given by Meyerhof and Lohmann (23), was used in these calculations. Experimental tests of the brain homogenates with HDP showed that the rate at which equilibrium was reached was very rapid and that the equilibrium constant was in the range given by Meyerhof and Lohmann. It should be noted that, in any case, the calculation can cause only a small error, since under the conditions prevailing the equilibrium is in favor of triose phosphate.

TABLE III

Na^+ Effect on Phosphohexokinase Reaction in Brain

These experiments were run in Thunberg tubes for 4 minutes at 38° under 5 per cent CO_2-95 per cent N_2. The tubes contained in a volume of 1.15 ml. the following: 2×10^{-3} M ATP, 2.5×10^{-3} M FMP, 4×10^{-3} M K iodoacetate, 2×10^{-2} M KF and the usual K^+-NH_4^+ buffers, and 4×10^{-3} M $MgCl_2$.

Amount of brain homogenate	NaCl (final concentration)	Change in inorganic P	Increase in triose P (alkali-labile)	Increase in HDP-P (calculated)	P transferred from ATP to fructose-6-P (calculated)	Q^* values
ml.	M	γ	γ	γ	γ	
0.05		−2.0	10.0	0.8	5.4	65.0
	0.07	−0.3	10.0	0.8	5.4	65.0
0.1	'	1.3	14.1	2.3	8.2	49.3
	0.07	2.3	14.9	2.5	8.7	52.4
0.15		3.5	19.4	4.3	11.9	47.8
	0.07	2.5	21.6	5.2	13.4	54.0

* Fructose-6-phosphate converted per mg. of dry weight per hour, expressed as microliters.

TABLE IV

Effect of NaCl on Coupled Phosphorylation

These experiments were carried out in Warburg vessels at 38° for 30 minutes under 5 per cent CO_2-95 per cent N_2 with a total volume of 1.15 ml. The reaction mixture contained 5×10^{-4} M ATP, 5×10^{-3} M HDP, 2×10^{-2} M K pyruvate, 1.4×10^{-2} M glucose, 4×10^{-3} M $MgCl_2$, 2×10^{-2} M KF, 4.6×10^{-4} M reduced glutathione, and the usual buffers.

Experiment No.	Brain homogenate	Added NaCl concentration	Q values		Ratio, P to lactic acid
			Inorganic P uptake	Lactate production	
	ml.	M			
1	0.10		66.1	100.4	0.66
		0.07	65.4	93.7	0.59
1	0.15		65.9	110.9	0.70
		0.07	67.0	99.5	0.67
2	0.10		51.9	96.8	0.54
		0.07	50.7	100.5	0.50
2	0.15		58.9	96.9	0.61
		0.07	58.9	97.1	0.61

The disappearance of inorganic phosphate reflects not only the oxidative reaction but also Reactions 3 and 4, whereby phosphate is transferred to glucose. The results of two experiments are shown in Table IV, expressed as Q values. The final column shows the ratio of phosphate uptake to lactate production. In both experiments 0.07 M NaCl caused a slight lowering of the phosphate-lactate ratio but the decreases are very small.

It will be noted that the rate of lactate production is high, yielding a $Q^{N_2}_{lactate}$ of 94 to 100 compared with values of 65 to 80 in glycolysis experiments. Since the over-all rate of this system is greater than that of the glycolytic system as a whole, it seems unlikely that the inhibition of glycolysis by Na$^+$ occurs through an effect upon this particular segment of the system.

Action of NaCl on Phosphopyruvate Transphosphorylase—The fourth phosphorylation reaction of the glycolytic system involves the transfer of phosphate from phosphopyruvate to AMP or ADP. Boyer *et al.* (6, 7) reported that K$^+$ stimulated this particular reaction in muscle homogenates. In one experiment there was also an indication that high concentrations of Na$^+$ were inhibitory to the reaction, although this point was not elaborated.

The effect of Na$^+$ upon the transfer of phosphate from phosphopyruvate

TABLE V

Effect of NaCl on Transfer of Phosphate from Phosphopyruvate to Adenylic Acid

These experiments were run in Thunberg tubes at 38° for 4 minutes under CO_2-N_2. The solution contained 1×10^{-3} M AMP, 91 γ of phosphopyruvate P, 4×10^{-3} M $MgCl_2$, 2×10^{-2} M KF, and the usual K$^+$-NH$_4^+$ buffers in a total volume of 1.15 ml.

Amount of brain homogenate	Added NaCl (final concentration)	Inorganic P increase	Decrease in phosphopyruvate P	Q values*	Inhibition by Na$^+$ of phosphopyruvate P decrease
ml.	*M*	γ	γ		*per cent*
0.1		1.2	8.6	51.8	77.9
	0.07	0.5	1.9	11.4	
0.15		9.7	27.8	111.6	61.9
	0.07	8.8	10.6	42.6	
0.2		19.2	38.8	116.8	41.0
	0.07	9.3	22.9	68.9	

* Phosphopyruvate P utilized, as microliters per hour per mg. of tissue.

to AMP at three levels of tissue is shown in Table V. Phosphopyruvate was determined as the phosphate fraction liberated by hypoiodite hydrolysis (24). The amount of phosphopyruvate reacting with AMP was considered to be equivalent to the amount of phosphopyruvate which disappeared. A very definite inhibition by the Na$^+$, ranging from 77.5 to 41.0 per cent, is shown. Some increase in inorganic phosphate occurred in all cases, although the changes do not occur in any predictable manner. Presumably the inorganic phosphate arises from the hydrolysis of ADP or ATP formed by transfer of phosphate from the phosphopyruvate and need not be considered in the calculations. The presence of fluoride prevents the conversion of phosphopyruvate to phosphoglyceric acid and also inhibits the Apyrase action to some extent.

In this experiment, as in others not shown, the extent of the reaction as

measured by phosphopyruvate utilization did not show a linear relationship to the amount of tissue used, suggesting that some uncontrolled factor is involved. In all cases, however, a Na$^+$ inhibition comparable to that of Table V could be demonstrated. The Q values are given for comparison with the other glycolytic reactions in Table V. It will be noted that the higher concentrations of tissue give a rate exceeding that of over-all glycolysis, suggesting that this reaction is not necessarily a side reaction.

The finding of an Na$^+$ inhibition of this reaction agrees with the previous observation of K$^+$ stimulation of the same reaction reported by Boyer et al. (6, 7). These investigators demonstrated the K$^+$ effect upon the reaction in an indirect manner by showing the stimulatory action of the ions upon a system which transferred phosphate from 3-phosphoglyceric acid to creatine with AMP as a carrier. By balance studies and other considerations, they reached the conclusion that the phosphopyruvate-

TABLE VI

Effect of KCl on Transfer of Phosphate from Phosphopyruvate to Adenylic Acid

Conditions as in Table V, except that 81 γ of phosphopyruvate P were used.
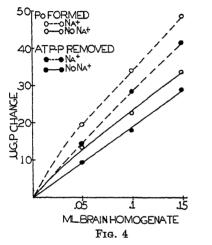
Amount of brain homogenate	Added KCl (final concentration)	Increase in inorganic P	Decrease in phosphopyruvate P	Q value
ml.	*M*	γ	γ	
0.1		−2.2	0	0
	0.07	−0.3	3.25	19.6
0.15		−0.3	0.8	3.2
	0.07	3.5	8.0	32.1
0.2		1.0	3.0	9.0
	0.07	4.0	18.5	55.7

AMP step was the reaction actually stimulated by K$^+$. Table VI shows the stimulatory effect of K$^+$ in a more direct fashion. The addition of 0.07 M KCl to a reaction mixture containing only sodium salts greatly stimulates the reaction. Presumably, the decrease in activity in this experiment as evidenced by the lower Q values was due to the fact that the brain homogenate was frozen overnight before use. The comparable experiments of Tables V and VI are those in which both Na$^+$ and K$^+$ were present; i.e., the last member of each pair in both tables.

Effect of Sodium Ions on Apyrase—In addition to the inhibitory effect of Na$^+$ upon the transphosphorylation reaction between phosphopyruvate and AMP, we have noted that Na$^+$ has still another effect on the glycolytic system, a stimulation of Apyrase action. Stimulation of Apyrase can cause a decreased rate of glycolysis, since the amount of ATP available for esterification reactions is diminished. As mentioned earlier, the inhibition by Na$^+$ was much more marked with homogenates than with extracts, suggest-

ing that one of the affected systems is removed by centrifugation. Meyer-
hof and Wilson (25) have shown that the greater portion of Apyrase is
absorbed on the particles of the brain homogenates which are removed by
centrifugation. Unpublished experiments from this laboratory confirm
this observation.

In Fig. 4, the stimulatory effect of Na^+ upon Apyrase is shown. The
results have been calculated in two ways: (a) the increase in inorganic
phosphate (Po) and (b) the disappearance of easily hydrolyzable phosphate
(ATP-P). Since there is a small endogenous production of inorganic
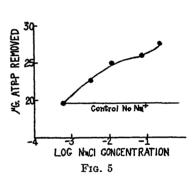
FIG. 4. Effect of NaCl on Apyrase activity of brain homogenates. Experiments
run in Thunberg tubes for 4 minutes under CO_2-N_2. Each tube contained, in addi-
tion to the brain homogenate, 1.5×10^{-3} M ATP, 4×10^{-3} M $MgCl_2$, and the usual
K^+-NH_4^+ buffers in a volume of 1.0 ml. Where indicated 0.07 M NaCl was added.

FIG. 5. Apyrase activity with varying NaCl concentration. 0.1 ml. of brain
homogenate used throughout. Other experimental conditions as described in Fig. 4.

phosphate, we have found the decrease in ATP to be a more reliable
estimate.

According to either method of calculation Na^+ stimulates the rate of
Apyrase activity at all concentrations of brain tissue employed. The
amount of stimulation varied somewhat from experiment to experiment,
as was the case with the inhibitory effect in glycolysis experiments, but the
results given in Fig. 4 are typical. The stimulation of Apyrase by Na^+
amounts to approximately 50 per cent when the three different tissue
levels are averaged, but it is probable that this loss of ester phosphate is
sufficient to influence the course of glycolysis markedly, since the system is

rather delicately balanced and small changes in the rate of a limiting reaction can be magnified when repeated cyclically.

In tissue studies not depicted here it has been found that the reaction rate of the Apyrase falls off after about 4 minutes, probably because of substrate limitations. The reaction mixture contained about 93 γ of hydrolyzable P calculated on a basis of two phosphate groups per molecule of ATP or 46.5 γ on the basis of conversion to ADP. At 4 minutes in Fig. 4 in the presence of Na$^+$, 43 γ of ATP-P had already been released. The amount of ATP used in these experiments is comparable to that in the glycolytic experiments, illustrating that Apyrase is sufficiently active under the conditions of glycolysis to destroy a major portion of the ATP in a very short time.

It was of interest to determine the effect of the concentration of Na$^+$ on the action of Apyrase. The results are shown in Fig. 5 in which micrograms of ATP-P hydrolyzed are plotted against the log of the NaCl concentration. Stimulation of Apyrase by Na$^+$ can be detected at a concentration of about 0.003 M. Since glycolysis was appreciably inhibited only at concentrations above this point, this experiment shows that Apyrase is at least as responsive to low concentrations of Na$^+$ as the entire glycolytic system. This observation supports the thesis that stimulation of Apyrase may constitute a major mechanism whereby Na$^+$ inhibits glycolysis.

In all of the foregoing experiments, effects of Na$^+$ were demonstrated by adding Na$^+$ to a reaction mixture which contained 0.004 M Mg^{++}, as is the case in the glycolytic system. As shown in Fig. 6, the action of Na$^+$ is conditioned by the presence of Mg^{++}. The results are plotted as Apyrase activity against varying MgCl$_2$ concentrations in the presence of 0.07 M NaCl and without NaCl. Surprisingly, the Na$^+$ effect was negligible when Mg^{++} was omitted and did not reach a maximum until a concentration of 0.004 M MgCl$_2$ was attained. Thereafter as the Mg^{++} concentration was increased, the activity declined in both cases in an essentially parallel fashion. It should be recalled that the 0.004 M MgCl$_2$ was present in the glycolytic experiments and in all of the foregoing experiments on Apyrase also. In a few experiments not shown here MnCl$_2$ was used to replace MgCl$_2$. Although Mn^{++} stimulated Apyrase activity to about the same extent as Mg^{++}, the addition of Na$^+$ did not enhance the effect to the same degree as it did with Mg^{++}.

The mechanism of the combined action of Mg^{++} and Na^{++} is not clear. Kielley and Meyerhof (26) have recently studied an Mg^{++}-stimulated ATPase from muscle and there have been other reports of Mg^{++}-stimulated Apyrases in brain (25) and *Escherichia coli* extracts (27). The decreasing activity with higher Mg^{++} concentrations may be due to the removal of ATP as the insoluble magnesium salt, since Mg ATP has a limited solu-

bility.   It is possible but unlikely that the Na$^+$ acts by affecting the solu·
bility of ATP.   It seems more probable that Na$^+$ is in some way influencing
the .enzyme itself, possibly by altering the physical state.   Meyerhof and
Wilson (28) have recently reported that brain Apyrase exhibits differen
properties toward certain inhibitors, depending on its physical state.

In Fig. 7 the effect of Na$^+$ upon the dephosphorylation of ADP is com·
pared with the action on ATP.   It is clear that Na$^+$ exerts a stimulatory
effect upon the removal of the second labile phosphate group as well a
the first, although the rate of action on ADP is somewhat lower, ever

FIG. 6   FIG. 7
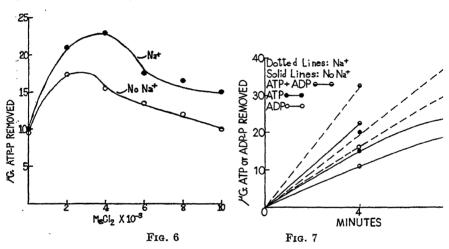
FIG. 6. Interaction of Mg^{++} and Na$^+$ on brain Apyrase.   0.1 ml. of brain homog
enate used.   Other experimental conditions as in Fig. 4.

FIG. 7. Activity of brain Apyrase on ATP and ADP.   The tubes contained 1.
$\times$ 10^{-3} M ATP, 3.0 $\times$ 10^{-3} M ADP, and 0.07 M NaCl when indicated.   Other condi
tions as in Figs. 4 to 6.

though the amount of hydrolyzable phosphate was the same in both experi
ments.   The mechanism of phosphatase action upon ADP in brain is no
known.   The possibilities exist that ADP is dephosphorylated via ATI
after a myokinase-like reaction, that ADP and ATP are dephosphorylated
by different phosphatases, or that a single enzyme attacks both substrates
It is interesting to note that the activity of the brain preparation upon ;
mixed substrate of ADP and ATP is almost equal to the sum of the activit}
upon the two substrates separately.   It should be mentioned that, witl
the short periods of incubation used in these studies, dephosphorylation o
AMP does not appear to be a significant factor.   Meyerhof and Wilsoı
(25) came to similar conclusions in their studies on brain Apyrase.

DISCUSSION

The relative importance of ADP and AMP as acceptors in the phospho-pyruvate transphosphorylase reaction is not clearly understood. On the basis of indirect evidence, Boyer *et al.* (7) came to the conclusion that ADP was superior to AMP as a phosphate acceptor in the phosphopyruvate reaction in muscle extracts. Kubowitz and Ott (29) reported that an enzyme isolated from human muscle catalyzed the transfer to ADP. Although the situation in brain may be similar, it must be noted that, in the absence of Na^+, the transfer to AMP by brain homogenates is more than adequate to account for glycolysis (Table V), making it difficult to dismiss this reaction from consideration.

In view of the above observations it is possible to postulate a mechanism for the inhibition of glycolysis by Na^+. The importance of an adequate supply of ATP for the maintenance of glycolysis is well known. In any system in which the ATP concentration is dependent upon a balance of synthetic and phosphatase reactions, a stimulation of the latter reactions may lead to a decreased rate or cessation of activity in the over-all system. The brain homogenate-glycolysis preparation seems to be rather delicately balanced in this regard and any increase in Apyrase activity may be expected to be reflected in decreased glycolysis. Since the dephosphorylation of ADP as well as ATP is promoted by Na^+, the production of AMP is enhanced. It is interesting to note that AMP and its deaminated derivative, inosinic acid, have been reported by Greenberg (30) to be inhibitory to glycolysis of brain preparations when present in a high concentration (0.001 M). Therefore, in addition to the loss of esterified phosphate through a heightened Apyrase activity, an inhibitory substance is formed at a greater rate. Likewise, the removal of AMP by rephosphorylation with phosphopyruvate is depressed by the Na^+. Greenberg (30) has shown that the AMP inhibition of glycolysis can be overcome by the presence of a large excess of a phosphate ester such as HDP, although an incubation period is required after the addition of HDP before the rate returns to normal. It would appear that there may be three interlocked effects of Na^+: a stimulation of the conversion of ATP to AMP, an inhibition of glycolysis by the AMP thus formed, and a decreased rate of removal of AMP by rephosphorylation. These three effects when taken together seem entirely adequate as an explanation for the marked inhibition of glycolysis by Na^+.

As mentioned earlier, the stimulatory effect of K^+ and the inhibitory effects of Na^+ are partially reciprocal in nature, at least as observed in the present experiments with brain and in the experiments by Boyer *et al.* (6, 7) with muscle preparations. It should be emphasized, however, that the addition of K^+ never completely reversed the effect of Na^+ and that, in-

versely, Na$^+$ inhibition was never complete in the presence of even small concentrations of K$^+$. The latter observation lends some support to the hypothesis that K$^+$ may play some rôle other than the reversal of Na$^+$ inhibition of certain glycolytic reactions as presented here. Muntz (10) has shown that the addition of small amounts of K$^+$ or NH$_4^+$ has a profound effect on the alcoholic fermentation by cell-free yeast preparations, even in the presence of relatively large concentrations of Na$^+$.

It would seem from data presented here that the Na$^+$ effect plays its most important rôle in homogenates, in which phosphate ester concentrations may be of prime importance in determining the course of the reactions and the Apyrase concentration is much smaller. The Na$^+$ inhibition can be demonstrated in extracts (Table I) however, and, as mentioned previously, Boyer et al. (6, 7) demonstrated the K$^+$-Na$^+$ effects on phosphopyruvate transphosphorylase with muscle extracts.

It is a pleasure to acknowledge the invaluable technical assistance of Mrs. Ellen Wolfe and the constant and stimulating interest of Dr. H. G. Wood.

SUMMARY

It has been shown that Na$^+$ inhibits glycolysis of homogenates of nervous tissue of the cotton-rat. The effect was reversed by HDP, although an imbalance of the phosphate esters persisted. The inhibition occurred at concentrations of Na$^+$ as low as 0.03 M and was due neither to the increased tonicity caused by the addition of NaCl nor to the chloride ions. The inhibition by Na$^+$ was less marked in extracts.

The effect of Na$^+$ upon various individual reactions of the glycolytic system was investigated. Hexokinase, phosphohexokinase, and the coupled oxidation-phosphorylation reactions were not significantly affected, but the transfer of phosphate from phosphopyruvate to AMP was inhibited and the dephosphorylation of ATP and ADP was stimulated.

The stimulation of Apyrase was shown to be operative at low levels of Na$^+$ and to be dependent upon the presence of Mg^{++}.

The significance of the effects of Na$^+$ upon the individual reactions has been discussed in relation to its effect upon glycolysis.

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THE STRONTIUM CONTENT OF HUMAN BONES*

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The metabolism of strontium in mammals has received considerable attention in recent years. One reason for such interest is that the metabolic behavior of strontium is similar to, though not identical with, that of calcium. In the study of the metabolism of several metals known to be deposited preferentially in the skeletal system, it became evident that a knowledge of the amounts of these metals normally found in human bones would be valuable.

Though many surveys of the mineral content of the bones of humans and lower animals have been made (1–9), as far as Sr is concerned, few workers have done more than allude to it as a trace element of wide-spread occurrence. Gerlach and Müller (4), in a survey by spectrographic methods of bone and soft tissues of birds and mammals, report that Sr is present in concentrations of 0.01 to 0.1 γ per gm. of fresh tissue. Rusoff and Gaddum (8) report that for new-born rats averaging 5.3 gm. the amount of Sr found in total body ash is around 1 γ.

This report presents the amounts of strontium found in representative bone samples taken from each of thirty-eight individuals arranged in five age groups.

Materials and Methods

The following age groups were arbitrarily defined and specimens from at least five individuals for each group were obtained at autopsy: fetus (over 10 cm.), new-born to 5 years, 5 to 20 years, 20 to 50 years, and over 50 years. Four samples were removed from each individual, parietal bone, rib, lumbar vertebrae, and diaphysis of femur (Table I). In addition, material was obtained from twelve cadavers, all of which had been in a state of preservation for approximately 35 years. Further information on these individuals was lacking, though it would appear that all were over 45 years of age at the time of death (Table II).

Sub-ject No.	Sex	Age	Reported cause of death	Parietal	Vertebrae	Rib	Femur
1	Fetus	13 cm.		0.015	0.016	0.018	0.015
2	"	13 "		0.017	0.016	0.016	0.017
3	"	12 "		0.017	0.019	0.018	0.016
4	"	16 "		0.016	0.016	0.016	0.015
5	"	18 "		0.015	0.015	0.018	0.017
Average				0.016	0.016	0.017	0.016
6	F.	5 mos.	Hydrocephalus	0.053*	0.055*	0.054*	0.055*
7	"	8 "	Bronchiolitis	0.019	0.018	0.018	0.018
8	M.	2½ yrs.	Retinoblastoma	0.028	0.026	0.029	0.022
9	"	3 "	Acute leucemia	0.025	0.025	0.028	0.018
10	F.	5 "	" "		0.019	0.022	0.024
11	M.	9 "	Trauma	0.023	0.017	0.017	0.019
12	"	13 "	Brain tumor	0.027	0.023	0.025	0.023
13	"	13½ "	Chronic nephritis	0.023	0.027	0.017	0.020
14	"	17 "	Trauma	0.026	0.018	0.021	0.027
15	"	17 "	"	0.025	0.024	0.023	0.023
16	"	27 "	Rheumatic heart disease	0.028	0.022	0.023	0.029
17	"	34 "	Trauma	0.022	0.019	0.020	0.022
18	"	34 "	Rheumatic heart disease	0.044*	0.023*	0.022*	0.028*
19	F.	38 "	Rheumatic heart disease	0.019	0.022	0.020	0.020
20	"	39 "	Cirrhosis	0.021	0.020	0.019	
21	M.	42 "	Cardiovascular accident	0.019	0.022	0.020	0.016
22	F.	51 "	Portal cirrhosis	0.021	0.023	0.024	0.021
23	"	54 "		0.024	0.021	0.020	0.023
24	M.	63 "	Gastric carcinoma	0.029	0.024	0.028	0.032
25	"	72 "	Cirrhosis	0.025	0.026	0.025	0.020
26	"	75 "	Pulmonary embolism	0.029	0.025	0.025	0.026
Average				0.023	0.022	0.022	0.022

* Average of three determinations on each of two separately weighed portions of the ashed bone specimen.

All the samples were cleaned of fascia and muscle, dipped in 0.1 N HCl, washed with distilled water, and ashed in a muffle furnace for 12 hours at 600°. The white residue was then ground to pass a 140 mesh sieve and a portion was mixed with special spectroscopic graphite powder and $CuSO_4 \cdot 5H_2O$, the internal standard material, in the ratio of 1 part bone ash, 2 parts graphite, and 9 parts $CuSO_4 \cdot 5H_2O$. About 8 mg. of the mixture were placed in a platform type electrode cut from $\frac{1}{4}$ inch special spectroscopic graphite rods; the electrode served as the lower, positive electrode in an Applied Research Laboratories Multisource discharge, corresponding

TABLE II

Per Cent Strontium in Ashed Bones of Cadavers in State of Preservation Since 1914

The statistical error of a single determination is within ±0.1 of its value.

Subject No.	Parietal	Rib	Femur
1	0.036	0.026	0.033
2	0.026	0.026	0.026
3	0.023	0.020	0.023
4	0.024	0.027	0.026
5	0.026	0.026	0.019
6	0.021	0.020	0.020
7	0.030	0.039	0.031
8	0.020	0.023	0.020
9	0.023	0.026	0.024
10	0.033	0.032	0.033
11	0.033	0.028	0.029
12	0.023	0.026	0.020
Average.........	0.027	0.027	0.025

to a direct current arc at 7 amperes. The exposure was terminated as soon as the sample was completely consumed, *i.e.*, after about 40 seconds. A 2 meter grating spectrograph was used with spectrum analysis No. 2 photographic film.

Standard samples similar to the bone samples were prepared by using specially purified $CaCO_3$ in place of bone ash. Various amounts of strontium, from 0.015 to 0.48 per cent by weight of $CaCO_3$, were added in the form of an aqueous solution of strontium chloride; the mixtures were then dried and put through a 140 mesh screen. These standard samples were used to establish the working curve shown in Fig. 1. The copper line at 4651.17 A was the internal standard; the strontium line at 4607.34 A was the analysis line. In order to cover a wide range of concentrations with

one set of exposure conditions and one pair of lines, a two-step filter was placed in front of the film at the position of the strontium line.   The carbon powder, the $CuSO_4 \cdot 5H_2O$, and HCl were each examined for residual strontium and in no case was any of this element detected under the conditions used in this work.   The spectrographic analysis of the $CaCO_3$ by the addition method showed that it contained about 0.004 per cent strontium. This was taken into consideration in the standard samples when the $CaCO_3$ was an added constituent.

Seven to ten determinations on each of six standard samples showed that
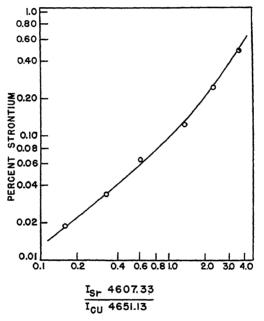
FIG. 1. Working curve for strontium

the standard deviation of the average of three determinations was 10 per cent of the amount present for the range of strontium concentrations found in the samples examined in the present work.   (To obtain such accuracy all unknowns were analyzed in triplicate.)

DISCUSSION

Perhaps the first obvious observation to be made is that the Sr content in almost all the individuals is relatively constant.   The lower values for fetal specimens might be explained on the basis that the processes of calcification have not been in operation as long in more mature specimens. Therefore, not only is the total inorganic content of the fetal bony struc-

tures low, but the opportunity for depositing "foreign" ions has been limited. Concerning the transmission of Sr to the fetus, the experiments of Pecher and Pecher (10) have demonstrated, for mice at least, that radio-strontium can migrate from the skeletal system of the pregnant animal to the fetus during the last stage of pregnancy. Even mothers' milk contains appreciable amounts of non-radioactive strontium (11). It is probable that continuously throughout life small amounts of Sr are ingested.

Although the foods consumed by humans during the 40 to 50 years prior to 1914 probably differed from what we today would call an average diet, such differences are not reflected by the Sr content in the bones of the 1914 individuals compared to the 1949 series.

Attention is drawn to the high Sr values for the bones of a 5 month-old hydrocephalic child (Table I, Subject 6). However, speculations as to possible correlation of the physiological disorder and the seemingly abnormal level of bone strontium are hardly justifiable on such meager data.

Within any given individual, there appear to be no gross differences in the amount of Sr deposition in the various bone tissues studied. In contrast, Pecher (12) has shown that the vertebrae of mice injected intravenously with Sr^{89} salts are more radioactive than other bony structures, indicating more rapid deposition in vertebral calcified tissue. It may well be that with the passage of time the normal dynamics of bone salt formation and dissolution bring about a more even distribution of such materials throughout the skeletal system.

Although the observations show some Sr present in all the bones examined, this is not evidence that Sr is an *essential* component of bone.

SUMMARY

1. Spectrographic analyses for strontium were made on samples of parietal, vertebral, rib, and femur bone ash from twenty-six individuals varying in age from fetus to 75 years. In addition, similar analyses were performed on parietal, rib, and femur samples obtained from twelve cadavers, all of which had been in a state of preservation since 1914.

2. The Sr content of all fetal specimens lay in the range of 0.015 to 0.019 per cent (average, 0.016 per cent). The range for all other specimens, save one, was 0.016 to 0.044 per cent (average, 0.024 per cent).

3. One hydrocephalic child showed an abnormally high bone Sr level (0.054 per cent).

4. Within any given individual there were no significant differences in Sr content between parietal, vertebral, rib, or femur bone, although the Sr level of all these specimens may have been high or low with respect to the average for all individuals studied.

5. The Sr content of the bones of the twelve individuals who died shortly

prior to 1914 was not significantly different from that found in the 1949 series. Thus, any major differences in dietary habits in the two time periods were not reflected by the present data.

6. It is concluded that Sr is a constant, though not necessarily essential, component of human bone.

The authors wish to express their gratitude to Dr. Thomas J. Haley and Dr. Moses A. Greenfield for their helpful advice.

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THE RELATION OF THE ELECTROLYTE COMPOSITION
OF PLASMA AND SKELETAL MUSCLE*

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Darrow and his associates (1, 2) have recently obtained evidence that a correlation exists between the concentration of serum bicarbonate, muscle potassium, and intracellular sodium from a study on rats subjected to procedures designed to produce a deficit of one of the ions sodium, chloride, or potassium. Thus, the serum bicarbonate concentration was found to vary directly with the intracellular sodium and inversely with muscle potassium when renal adjustment was attained in the presence of a deficiency of sodium, chloride, or potassium. As an example, when a potassium deficiency was induced by maintaining rats on a diet low in potassium, or by the repeated injection of desoxycorticosterone acetate (DCA), the loss of muscle potassium and gain of intracellular sodium were found to be accompanied by an elevation of the serum bicarbonate concentration. In addition, the elevated serum bicarbonate concentration was found to be associated with a decreased serum chloride concentration.

Several years ago one of the present authors (E. M.) and others (3) noted that dogs subjected to repeated injections of DCA exhibited a striking reduction of muscle potassium, a gain of intracellular sodium, and a slight decrease of the plasma chloride concentration. In this latter study there was found to be a tendency toward an increased plasma bicarbonate concentration (not reported at the time, but see below); however, there appeared to be no clear relationship between the changes of the muscle sodium and potassium and the plasma bicarbonate level. In this respect the observations on dogs with potassium deficiency differed from those on rats.

The experiments included in this paper represent further studies on dogs and rats with potassium deficiency induced by the administration of DCA or by maintaining the animals on a diet low in potassium. They were undertaken for the following purposes: (1) to observe and compare the relationship of the electrolyte composition of plasma and skeletal muscle of dogs and rats, and (2) to observe the influence of altering the dietary

intake of sodium and chloride on the changes of the electrolyte composition of plasma and skeletal muscle of potassium-deficient animals.

Methods

In the experiments on dogs, young adult animals were placed on a constant diet (4) for at least 2 weeks before the experiments were undertaken. The animals which were maintained on the low potassium diet were given the basal diet without the daily supplement of potassium chloride. The dietary supply of sodium and chloride was altered by appropriate adjustment of the daily supplement of sodium citrate or sodium chloride. Certain of the animals received DCA[1] (0.5 mg. per kilo of body weight) daily by subcutaneous injection for a period of 3 weeks.

In the experiments on rats, young adult males of the Wistar strain, of a uniform age and weight, were placed on a control diet for a period of 7 days. The control diet consisted of commercial casein 24 per cent, glucose 62 per cent, corn oil 8 per cent, and a salt mixture (5) 6 per cent. In addition all animals received daily (6 days a week) 2 drops of a vitamin supplement which furnished the following water-soluble vitamins in the amounts indicated: thiamine hydrochloride 25 γ, riboflavin 30 γ, nicotinamide 25 γ, pyridoxine hydrochloride 25 γ, calcium pantothenate 200 γ, choline chloride 10 mg., and inositol 3 mg. Twice weekly all animals received 2 drops of a cod liver oil-vitamin E supplement which furnished 200 U. S. P. units of vitamin A, 20 U. S. P. units of vitamin D, and 1 mg. of α-tocopherol. The salt mixture (5) of the diet was altered in composition with respect to sodium, potassium, and chloride when the rats were placed on experiment. Some of the animals received daily subcutaneous injections of DCA (2 mg. per day) for 14 days. The results of the analyses of the several diets employed are shown in Table I.

Blood from dogs was withdrawn under oil by direct puncture of the femoral artery, placed under oil in a special centrifuge tube containing heparin ("liquaemin," Organon, Inc.) to prevent clotting, and centrifuged at once for the plasma analyses. Skeletal muscle (lumbar portion, sacrospinalis) was obtained under sodium pentobarbital anesthesia as quickly as possible following the collection of the blood and sampled for the several analyses in the manner previously described (6). The rats were placed under sodium pentobarbital anesthesia and arterial blood was collected under oil by direct puncture of the left ventricle. The gastrocnemius muscles were then removed. In order to provide adequate samples for the several analyses, equal aliquots of plasma separated under oil from two rats in the same group (control or experimental) were combined under oil.

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[1] We are indebted to the Ciba Pharmaceutical Products, Inc., for the generous supply of "percorten" which was employed in these experiments.

The gastrocnemius muscles from the two rats were also combined and sampled. In presenting the results of the analysis of plasma and muscle of rats, therefore, the designation in Table II of the number of animals employed in a given experiment represents the number of animal "pairs."

The following determinations were made on the plasma: water, sodium, potassium, chloride, and total CO_2; on the muscle, water, chloride, sodium, potassium, and total fat. The chemical methods for the analysis of plasma obtained from both dogs and rats were the same as those employed in previous studies (7), with the exception that the plasma for the determinations of sodium and potassium was ashed as described by Eichelberger and Roma (8). Chlorides were determined on dog muscle by the wet ashing method described by Hastings and Eichelberger (9). The ashing procedure described by Eichelberger (10) was employed for the determination of sodium and potassium in dog muscle. All of the other procedures on dog

TABLE I

Composition of Diets for Rats per 100 Gm.

Type of diet	Na	K	Cl
	mM	*mM*	*mM*
Control......................................	32	11.5	27
Low potassium			
Normal Na and Cl......................	32	0.1	27
" " high Cl.....................	32	0.1	56
" " low Cl.....................	32	0.1	3
Low Na, high Cl...................... .	14	0.1	43

muscle were the same as those employed in previous studies (7). The chemical determinations on rat muscle were carried out on the dry fat-free tissue remaining after the determinations of water and fat content. This residue was finely ground with a Wiley laboratory mill,[2] intermediate model, with 20 mesh sieve, and kept in a weighing bottle. Before sampling, the powder was dried to a constant weight at 100–105°. Chlorides were determined on weighed aliquots of the powder according to the method of Eichelberger and Bibler (11). For the determinations of sodium and potassium weighed aliquots of the powder were first ashed as described by Eichelberger and Bibler (11). The ash was dissolved in 5 cc. of 1 N HCl and transferred to a 25 cc. volumetric flask with hot redistilled water, and then treated in the same manner as the solution of the ash obtained from dog muscle.

The data obtained on plasma are expressed in terms of 1000 gm. of

[2] Arthur H. Thomas Company, Philadelphia.

plasma water.   The plasma bicarbonate was calculated from the figure for plasma total CO_2 by the Henderson-Hasselbalch equation, assuming a constant pH of 7.4.   The data obtained on muscle are presented in terms of 100 gm. of fat-free solids.   For comparative purposes the intracellular sodium of both dog and rat muscle was calculated in the manner described by Darrow et al. (2) for rat muscle, as follows:

$$\frac{(Cl)_m - 1}{[Cl]_e} = (H_2O)_e$$

$$(Na)_m - (H_2O)[Na]_e = (Na)_i$$

in which $(Cl)_m$ and $(Na)_m$ are, respectively, total tissue chloride and sodium per 100 gm. of fat-free solids, $(H_2O)_e$ is the extracellular water per 100 gm. of fat-free solids, and $(Na)_i$ is the intracellular sodium per 100 gm. of fat-free solids. $[Cl]_e$ and $[Na]_e$ are, respectively, the concentrations of chloride and sodium in an ultrafiltrate of plasma, calculated from their corresponding plasma concentrations per 1000 gm. of plasma water and an average Donnan factor of 0.96.   The constant, 1, appearing in the first equation is an average correction for chloride that apparently does not react like extracellular chloride (12).

Results

Electrolyte Content of Plasma and Skeletal Muscle of Rats with Potassium Deficiency

The results of the analysis of plasma and skeletal muscle of rats maintained on the control diet and on a diet low in potassium (Group I) and of rats receiving DCA (Group II) are presented in Table II.   It will be seen that in each subgroup of the animals placed on the potassium-deficient diet for a period of 5 weeks there was a loss of muscle potassium and a gain of intracellular sodium.   Further, the changes in muscle composition were accompanied by an elevated plasma bicarbonate concentration and by a fall of the plasma chloride.   It should be pointed out that the altered plasma composition occurred in the presence of adequate dietary sodium and chloride.   On the other hand, it will be noted that, while the loss of muscle potassium was similar in the several subgroups of animals, the gain of intracellular sodium was somewhat less in those animals receiving diets containing chloride in excess of sodium.   In addition, the average increase of the plasma bicarbonate concentration and the fall of plasma chloride were also found to be less in these same animals.

The results of the analysis of plasma and muscle obtained from rats receiving DCA for a period of 14 days revealed changes similar to those encountered in animals maintained on a potassium-deficient diet.   Thus, a

plasma bicarbonate increase and a plasma chloride decrease were found to accompany the deficit of muscle potassium and gain of intracellular sodium.

The observation of an increased plasma bicarbonate concentration in the presence of a loss of muscle potassium and a gain of intracellular sodium in potassium-deficient rats is in agreement with the finding of Darrow and his coworkers (2).

TABLE II

Average Electrolyte Content of Plasma and Skeletal Muscle of Rats Maintained on Diets Deficient in Potassium and of Rats Receiving Desoxycorticosterone Acetate

	Plasma per kilo water				Muscle per 100 gm. fat-free solids				
	Na	K	HCO₃	Cl	H₂O	Cl	Na	K	(Na)ᵢ
Group I. Control and low K diets for 5 wk. period									
	m.eq.	*m.eq.*	*m.eq.*	*m.eq.*	*gm.*	*m.eq.*	*m.eq.*	*m.eq.*	*m.eq.*
Control diet (3)*..............	156.1	4.1	26.9	106.8	325.6	5.1	9.3	45.3	3.8
Low potassium diet									
Normal Na, normal Cl (3)...	158.4	2.1	43.7	87.7	320.8	4.3	18.7	32.5	13.2
" " high Cl (3).....	157.1	2.2	36.4	98.1	320.5	5.2	17.0	33.5	10.9
" " low Cl (3)......	154.0	2.1	41.8	88.9	324.3	3.6	18.8	33.4	13.0
Low Na, high Cl (3)........	156.3	2.5	36.2	98.8	311.5	4.6	17.1	32.4	11.8
Group II. Control and low K diets, DCA† administration for 2 wk. period									
Control diet (2)..............	152.3	4.5	29.8	103.7	324.7	5.6	8.9	45.3	2.7
" " + DCA (2).......	158.1	3.4	42.4	91.3	317.0	5.5	15.7	35.1	8.6
Low potassium‡ (2)...........	155.6	3.1	33.0	102.5	314.0	5.7	12.9	38.0	6.3
" " ‡ + DCA (2)...	158.8	2.7	45.1	90.5	316.5	5.6	16.8	33.1	9.3

* The numbers within parentheses indicate the number of pairs of rats.
† Daily subcutaneous injection of 2 mg. per rat.
‡ Low potassium and normal sodium and chloride diet.

Electrolyte Content of Plasma and Skeletal Muscle of Dogs with Potassium Deficiency

Following Administration of Desoxycorticosterone Acetate—Table III presents the results of the analysis of plasma and muscle obtained from dogs following the injection of DCA (1 mg. per kilo of body weight daily) for a period of 14 days and from animals 14 days after the administration of DCA had been discontinued. These data, with the exception of the values for the plasma bicarbonate concentration, were included in a previous study (3). In comparison with the control values, it will be seen that following the administration of DCA the decrease of muscle potassium and the gain of intracellular sodium were accompanied by an average decrease of the

plasma chloride concentration of 4.3 m.eq. per kilo of water and by an average increase of the plasma bicarbonate concentration of 3.2 m.eq. per kilo of water. The maximum plasma bicarbonate concentration encountered in this group of animals was found to be 29.8 m.eq. per kilo of water and was accompanied by a muscle potassium of 21.0 m.eq. per 100 gm. of fat-free solids (a minimum value for the group) and by an intracellular sodium of 12.4 m.eq. On the other hand, the minimum plasma bicarbonate concentration was 20.3 m.eq. per kilo of water and was associated with a muscle potassium of 29.2 m.eq. per 100 gm. of fat-free solids and with an intracellular sodium of 9.9 m.eq.

The data presented in Table III reveal that a marked alteration of the

TABLE III

Average Electrolyte Content of Plasma and Skeletal Muscle of Dogs Following Administration of Desoxycorticosterone Acetate and after Its Discontinuance

	Plasma per kilo water				Skeletal muscle per 100 gm. fat-free solids				
	Na	K	HCO₃	Cl	H₂O	Cl	Na	K	(Na)ᵢ
	m.eq.	m.eq.	m.eq.	m.eq.	gm.	m.eq.	m.eq.	m.eq.	m.eq.
Controls (40)	156.0	3.3	22.1	118.6	321.7	8.3	12.4	36.3	3.6
S.d.	2.8	0.6	2.7	3.0	14.8	1.2	1.7	2.6	1.2
Following DCA (9)	156.5	2.1	25.3	114.3	317.3	7.3	19.2	27.9	9.9
S.d.	3.1	0.4	3.1	2.6	17.9	0.7	2.6	4.1	2.6
After DCA discontinuance (3)†	159.2	2.2	22.0	117.1	321.5	8.8	19.9	25.9	10.1
After DCA discontinuance (5)‡	156.3	2.6	22.4	116.5	327.2	8.5	17.1	31.4	7.8

* 2 week periods. 1 mg. per kilo of body weight of DCA was administered daily. The numbers within the parentheses indicate the number of animals.

† High sodium and low potassium diet.

‡ Low sodium and moderately high potassium diet.

muscle composition with respect to potassium and sodium continued to be present in the dogs observed 14 days after the administration of the DCA had been discontinued. On the other hand, the average plasma bicarbonate and chloride concentrations were found to approach the corresponding control values.

The results of these experiments indicated, therefore, that potassium deficiency induced by the administration of DCA to dogs maintained on a low potassium diet is accompanied by a tendency toward an increased plasma bicarbonate concentration. However, when compared with the change observed in rats, the average increase of the plasma bicarbonate concentration was found to be relatively small. In addition, the level of

plasma bicarbonate appeared to bear no clear relationship to the changes of muscle potassium and intracellular sodium.

From the data presented in Table II it is seen that with rats the rise of the plasma bicarbonate concentration was associated with a more or less equivalent but reciprocal change of the plasma chloride concentration. With dogs, the plasma bicarbonate and chloride concentrations were altered in a similar manner, but the magnitudes of the changes were less. These observations suggest the possibility that the increase of the plasma bicarbonate concentration is dependent upon the extent of the plasma chloride decrease and, further, that the plasma chloride concentration can be better maintained in dogs than in rats. It was of interest, therefore, to carry out additional experiments on dogs. In one experiment, in order to confirm the previous findings on dogs, a group of animals was maintained on a low potassium and a normal or high sodium chloride diet and DCA was given (0.5 mg. per kilo of body weight daily) for a period of 3 weeks. In a second experiment, the effect of restricting the intake of chloride as well as potassium was observed. For the latter purpose two dogs were placed on a diet low in potassium and chloride but normal with respect to sodium, and DCA was administered in the same amount and for the same period of time as with the other animals.

From the data presented in Table IV, it will be seen that the average changes of the electrolyte composition of the plasma and muscle encountered in the group of eleven dogs receiving DCA for a period of 21 days were similar to those observed in the animals reported above. An examination of the data obtained from dogs treated in this manner revealed that the changes in muscle composition with respect to potassium and intracellular sodium were of a magnitude similar to those observed in rats. On the other hand, the results of the analysis of the plasma obtained from these dogs gave ample evidence that the changes of the bicarbonate and chloride concentration were less pronounced than those encountered in rats. In this connection, the results obtained on the two dogs which received DCA while restricted in their dietary intake of potassium and chloride are of special interest. From the data obtained on these animals, presented in Table IV, it will be seen that the average plasma bicarbonate concentration was increased to 41.3 m.eq. and was accompanied by a fall of the plasma chloride to 97.1 m.eq. per kilo of water. The chemical analysis of muscle revealed that the potassium was reduced to an average value of 25.1 m.eq. and that the intracellular sodium was increased to an average value of 17.1 m.eq. per 100 gm. of fat-free solids. Thus, with a dietary restriction of chloride as well as of potassium, the administration of DCA led to a marked alteration of the electrolyte composition of muscle and to changes of the plasma bicarbonate and chloride concentration which were of magnitudes even greater than those observed in potassium-deficient rats.

Following Dietary Restriction of Potassium—In commenting on the data presented in Table II, it was pointed out that with rats placed on a low potassium diet for a period of 5 weeks there occurred an increase of the plasma bicarbonate concentration and a fall of plasma chloride, even though the animals' diet was normal with respect to sodium and chloride. From other experiments it was found that a rise of the plasma bicarbonate

TABLE IV

Average Electrolyte Content of Plasma and Skeletal Muscle of Dogs Maintained on Diets Deficient in Potassium and of Dogs Receiving Desoxycorticosterone Acetate

	Days on diet	Plasma per kilo water				Skeletal muscle per 100 gm. fat-free solids				
		Na	K	HCO₃	Cl	H₂O	Cl	Na	K	(Na)ᵢ
		m.eq.	*m.eq.*	*m.eq.*	*m.eq.*	*gm.*	*m.eq.*	*m.eq.*	*m.eq.*	*m.eq.*
Normal diet (5)*	21	156.8	4.5	24.5	115.4	314.8	4.9	7.8	42.1	2.9
Low potassium + DCA† (11)	21	161.1	2.7	28.1	113.9	325.7	6.8	14.8	34.2	7.1
S.d.		3.2	0.6	3.1	3.9	20.9	1.0	4.3	5.3	3.6
Low potassium-low chloride, normal sodium (2)	21	153.4	3.5	23.8	113.3	328.4	6.5	11.1	37.8	4.3
Same + DCA† (2)	21	155.1	1.8	41.3	97.1	324.9	5.8	24.2	25.1	17.1
Low potassium-low chloride, normal sodium (2)	84	159.3	3.0	28.7	106.5	310.3	4.2	8.8	39.1	4.4
Same (1)	84		3.7	25.3	98.0					
After NaCl intake‡		163.8	3.5	18.7	120.7	290.0	4.5	8.0	37.9	3.6
Low potassium-low chloride, normal sodium (2)	84	162.2	3.1	31.9	104.7					
After NaCl and KCl intake§		164.3	4.1	20.1	115.3	317.5	5.9	9.7	40.4	2.6

* The numbers within the parentheses indicate the number of animals.
† 0.5 mg. per kilo of body weight of DCA was administered daily.
‡ Diet supplemented with 1 gm. of NaCl per kilo of body weight per day for 3 days.
§ Diet supplemented with 0.4 gm. of NaCl and 0.69 gm. of KCl per kilo of body weight per day for 3 days.

concentration and a fall of the plasma chloride could be demonstrated with rats on the same dietary regimen for as short a period as 7 days. On the other hand, it has been our experience that the electrolyte composition of muscle and plasma of dogs receiving a low potassium and a normal or high sodium chloride diet for a period of 21 days shows relatively little alteration from the normal.

In Table IV are presented the results of the analysis of plasma and muscle of dogs maintained on a diet low in potassium and chloride but normal in

sodium content for periods of 21 and 84 days. It will be seen that the two dogs which received the diet for 21 days exhibited an average loss in muscle potassium of 4.3 m.eq. and an average gain in intracellular sodium of 1.4 m.eq. per 100 gm. of fat-free solids when compared with the corresponding average control values. The average values for potassium and intracellular sodium of muscle from the two dogs placed on the same diet for 84 days revealed deviations from the normal which approximated those observed in the animals above. A similar comparison of the plasma composition revealed that both the plasma bicarbonate and chloride were reduced slightly following the shorter dietary period. On the other hand, with the more prolonged dietary restriction of potassium and chloride there was a definite tendency toward a reduction of the plasma chloride concentration and an increase of the plasma bicarbonate.

The changes of the electrolyte composition of plasma and muscle exhibited by the dogs following the prolonged dietary deficiency of potassium and chloride were similar in direction to those observed in rats when placed on a low potassium diet, but were of a smaller magnitude. In order to observe the effect on the plasma and muscle of supplementing the diet with sodium and potassium chloride, additional experiments were carried out on dogs after they had been maintained on the diet low in potassium and chloride for a period of 84 days. For this purpose the diet of one dog was supplemented with 1 gm. of sodium chloride per kilo of body weight per day for 3 days, and the diet of two other dogs was supplemented with 0.4 gm. of sodium chloride and 0.69 gm. of potassium chloride per kilo of body weight daily for 3 days. From the data presented in Table IV, it will be seen that the supplementation of the diet with sodium chloride resulted in an increase of the plasma chloride from 98.0 to 120.7 m.eq. and a fall of the plasma bicarbonate from 25.3 to 18.7 m.eq. per kilo of water. On the other hand, the levels for muscle potassium and intracellular sodium apparently underwent no change, since these values were found to approximate those observed in the animals studied at the close of the dietary period. In the two dogs whose diet was supplemented with sodium chloride and potassium chloride there was also found to be a prompt increase of the plasma chloride from an average of 104.7 to 115.3 m.eq. and a fall of the plasma bicarbonate concentration from an average of 31.9 to 20.1 m.eq. per kilo of water. In this case the chemical analysis of the muscle revealed a tendency toward restoration of normal levels of potassium and intracellular sodium.

The results of these experiments indicate that with dogs subjected to rather prolonged periods of dietary restriction of potassium and chloride there is a resistance to marked alterations of the electrolyte composition of plasma and muscle. Under the latter experimental conditions, there was found to be a tendency toward a loss of muscle potassium and a gain of

intracellular sodium, while the plasma bicarbonate concentration tended to be increased and the plasma chloride to be reduced. However, the concentrations of plasma chloride and bicarbonate can apparently be restored to normal in such dogs by dietary supplementation with sodium chloride, even though the muscle composition with respect to potassium and intracellular sodium remains distorted.

DISCUSSION

From the work carried out in a number of laboratories it is well established that potassium deficiency in rats induced by the dietary restriction of this element or by the repeated injection of DCA leads to a deficit of muscle potassium and a gain of intracellular sodium. The recent observation by Darrow and his coworkers (2) that these changes in muscle composition are associated with an increased plasma bicarbonate concentration is confirmed in the present study. In this connection, the observations of Hegnauer (13), who studied the effect of a low potassium diet and of DCA on the electrolyte content of rat blood and muscle, are of interest. This author noted a diminished plasma chloride concentration, particularly in the animals receiving DCA. Although CO_2 determinations were not carried out, Hegnauer predicted on the basis of a fall of the plasma chloride concentration unaccompanied by a decrease of plasma cation that the alkali reserve would be found to be increased. It is true that the plasma cation concentration undergoes a relatively small change. Consequently, it would appear that the simultaneous fall of the plasma chloride represents an important factor determining the rise of the plasma bicarbonate concentration encountered in potassium-deficient animals. In the present study, a pronounced fall of the plasma chloride concentration, accompanied by an approximately equivalent increase of the plasma bicarbonate concentration, was observed in rats maintained on a diet low in potassium and normal with respect to sodium and chloride. However, when the low potassium diet was altered to contain chloride in excess of sodium, the fall of the plasma chloride concentration and the accompanying increase of the plasma bicarbonate concentration were found to be of a smaller magnitude, even though there was an equal deficit of muscle potassium.

A comparison of the results of the analysis of plasma and muscle of dogs with those obtained with rats under comparable experimental conditions leads to the impression that the plasma chloride concentration can be better maintained in dogs. For example, when DCA was administered to dogs receiving a low potassium but normal or high sodium chloride diet, the changes of muscle potassium and intracellular sodium were found to be of a degree comparable to those observed in potassium-deficient rats. However, the plasma chloride deficit was less in dogs than in rats, and the ac-

companying increase of the plasma bicarbonate concentration was also less. Thus, with administration of DCA to dogs on a diet deficient in potassium, but adequate with respect to chloride, a tendency toward stabilization of the plasma chloride concentration was observed. In contrast, when the diet was made deficient in chloride as well as potassium, the administration of DCA was found to lead to a striking fall of the plasma chloride concentration and an approximately equivalent increase of the plasma bicarbonate concentration, in addition to the marked alteration of the muscle composition with respect to potassium and sodium.

The results of the present experiments, dealing primarily with the effects of a deficiency of potassium, appear inadequate for a discussion of the following question: Do the changes of the plasma bicarbonate and chloride levels constitute an adjustment in response to an altered muscle composition with respect to potassium and sodium, or, do the changes in the composition of the plasma and muscle represent simultaneous processes related to the renal excretion of specific anions and cations? Further work is now in progress on this problem.

SUMMARY

A study was made of the electrolyte composition of plasma and skeletal muscle of rats and dogs with potassium deficiency induced by the administration of DCA and by maintenance on a low potassium diet. The effect of altering the dietary intake of sodium and chloride was also studied. The various findings may be briefly summarized as follows:

1. Potassium deficiency in rats led to a loss of muscle potassium and a gain of intracellular sodium, and, in agreement with the observations of Darrow et al., these changes in muscle composition were found to be accompanied by an increased concentration of plasma bicarbonate. Evidence was obtained that a simultaneous fall of the plasma chloride concentration represented an important factor determining the extent of the plasma bicarbonate increase, since the total cation concentration of the plasma was found to undergo only a relatively small change.

2. Deficits of muscle potassium and increases of intracellular sodium of magnitudes similar to those observed in rats were encountered in dogs when potassium deficiency was induced by the administration of DCA to animals maintained on a diet low in potassium but normal or high with respect to sodium and chloride. In these dogs, however, the fall of the plasma chloride concentration and the rise of the plasma bicarbonate concentration were found to be much less than were observed in the rats.

3. The latter observation lends support to the belief that the plasma chloride concentration was better maintained in dogs than in rats when potassium depletion was induced in the presence of adequate dietary chlo-

ride.   When DCA was administered to dogs receiving a diet low in chloride as well as potassium, the changes in muscle composition were accompanied by a striking deficit of the plasma chloride concentration and by a marked increase of the plasma bicarbonate concentration.

4. Dogs receiving a low potassium, low chloride but normal sodium diet for a period of 12 weeks exhibited some deficit of muscle potassium and gain of intracellular sodium, while there was a tendency toward a decrease of the plasma chloride and an increase of the plasma bicarbonate concentration.   The supplementation of the diet with sodium chloride led to a prompt increase of the plasma chloride concentration and a fall of the plasma bicarbonate, without any further change in muscle potassium or intracellular sodium.

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THE OXIDATION OF HEXANOIC ACID AND DERIVATIVES BY LIVER TISSUE IN VITRO*

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Experiments with labeled octanoic acid have shown that liver slices cleave the fatty acid to 2-carbon fragments which condense to form acetoacetate (1–3). The earlier findings (1, 2) of an equal distribution of the isotope between the carboxyl and carbonyl groups of acetoacetate after utilization of carboxyl-labeled octanoate indicate a *random* recondensation of 2-carbon fragments, while later findings do not support *random* recondensation and suggest two types of 2-carbon fragments (4, 5).

In the case of the 6-carbon hexanoic acid, the analytical work of Jowett and Quastel (6) and Leloir and Muñoz (7) indicates the formation of only 1 mole of acetoacetate from hexanoate rather than the 1.5 moles as required by a recondensation mechanism. The *in vivo* isotope experiments of Morehouse and Deuel (8) also do not favor recondensation in acetoacetate formation from hexanoate. The recent work of Crandall and coworkers (4, 5), however, supports the same recondensation mechanism for octanoate and hexanoate.

None of these studies reveals the detailed course of fatty acid oxidation or the nature of the oxidized intermediates formed prior to cleavage of the carbon chain. Several isolated reports (9–23) have dealt with the metabolism of a variety of possible fatty acid oxidation intermediates, but these have not been specifically devised to reveal the rate and extent of acetoacetate formation from the derivatives as compared to the parent fatty acid.

Previous studies in this laboratory (10) demonstrated the formation of 1 mole of acetoacetate from 1 mole of β,δ-diketohexanoate by liver homogenates and a purified liver enzyme. This possible intermediate and a number of other unsaturated, hydroxy, and keto derivatives of hexanoate have been utilized in the present study. Acetoacetate was stable in the washed liver particle system employed, and this system was therefore

ideally adapted to quantitative evaluation of substrate disappearance and product formation. Hexanoate and its derivatives are particularly suitable for study of the recondensation mechanism since a yield of 1.5 moles of acetoacetate from a 6-carbon substrate is absolute evidence for such a mechanism. Specific analytical methods for acetoacetate (10) and diketohexanoate (24) were previously developed, and methods for 2-Δ-hexenoic and sorbic acids are reported in this paper.

Chemicals—2-Δ-Hexenoic acid was synthesized by the method of Boxer and Linstead (25). Ethyl β-hydroxyhexanoate (26) and ethyl β-hydroxy-γ-hexenoate (27, 28) were prepared by the Reformatsky reaction by allowing ethyl bromoacetate to react with butyraldehyde and crotonaldehyde respectively. The corresponding free acids were isolated through the barium salts. β-Hydroxy-γ-hexenoic acid, for which no record of physical properties was found, is a white crystalline solid melting at 46.5°, soluble in water, ethanol, and ethyl ether. Upon refluxing with 20 per cent alkali this acid was converted to sorbic acid, m.p. 134°.

α,β-Dihydroxyhexanoic acid was synthesized by oxidation of 2-Δ-hexenoic acid with silver chlorate and osmic acid according to Braun (29). Sorbic and hexanoic acids, gifts of the Commercial Solvents Corporation, were purified by recrystallization and distillation respectively. Triacetic acid was prepared as previously described (30). Ethyl β-ketohexanoate was prepared according to Blaise (31) and Decombe (32) and purified through the magnesium salt (33). Fresh solutions of β-ketohexanoic and acetoacetic acids, free of ethanol, were prepared from the ethyl esters by the method of Krebs and Eggleston (34). Adenosine triphosphate was prepared from rabbit muscle by the method of Dounce *et al.* (35).

Liver Enzyme System—This was prepared by a method similar to that of Potter (36). Rats were fasted overnight, decapitated, the liver chilled, and a 10 per cent homogenate prepared in cold 0.85 per cent sodium chloride with a mechanical homogenizer (37). The homogenate was centrifuged at 5° in a refrigerated centrifuge, and the supernatant fluid was saved for preparation of "heat-treated liver extract." The particulate fraction was washed three times with cold 0.85 per cent saline, equal in volume to that of the original homogenate, by successive suspension of the insoluble fraction and centrifugation in the cold. The washed liver particles were finally suspended in saline such that 1 ml. contained the equivalent of 0.4 gm. of original liver, and the suspension was used immediately.

The supernatant fluid from the first centrifugation of the homogenate was heated in a boiling water bath for 5 minutes, centrifuged, and the clear yellow supernatant fluid was used as heat-treated liver extract.

Oxygen uptake measurements were made at 30° in 15 ml. Warburg flasks containing 3.1 ml. of reaction mixture and 0.2 ml. of alkali in the center cup. In these experiments the chilled enzyme solution was placed in the side arm of the flask and kept at 0–5° until the flask was equilibrated for 5 minutes in the 30° bath prior to manometric measurements. In other experiments the enzyme was placed in the main compartment of the flask and kept cold before incubation in the 30° water bath. A period of 40 to 60 minutes usually sufficed for complete oxidation of 3 μM of hexanoic acid; 60 minutes incubation were generally employed for experiments in which oxygen consumption was not measured.

Analytical Methods—Acetoacetic acid was estimated by the specific vanillin procedure previously described (10). β-Ketohexanoic acid was estimated by the difference in analytical values obtained with the aniline citrate method for decarboxylation of total β-keto acids (34) and the vanillin method (10) for acetoacetic acid. β-Hydroxybutyric acid was measured by the method of Behre (38), citric acid by a modification (39) of the pentabromoacetone procedure (40), acetaldehyde by the p-hydroxydiphenyl reaction (41), and triacetic acid by the o-phenylenediamine color reaction (24).

In the experiments with whole liver homogenate, hexanoate disappearance was measured by steam distillation of the acid and titration (10). 85 to 92 per cent of hexanoic acid was recovered, and the analytical results reported are corrected for this recovery. In washed liver system, in which lower concentrations of hexanoate were necessarily used, disappearance of the acid was estimated indirectly from the oxygen uptake. The oxygen consumption was considered to be a valid measure of hexanoate oxidation only when there was no oxygen uptake in the absence of the hexanoate, or when fatty acid oxidation was complete and the oxygen consumption in the absence of hexanoate was less than 10 per cent of that found for complete oxidation. In calculating ratios of hexanoate disappeared to acetoacetate production, the amount of acetoacetate formed in the absence of hexanoate was deducted from that formed in the presence of the fatty acid. Similar figures of control oxygen consumption were not deducted, however, since it was found that hexanoate depresses the endogenous oxygen consumption and that acetoacetate formation agreed with the theoretical oxygen consumption without correction of the latter.

Sorbic and 2-Δ-hexenoic acids were separated from metaphosphoric acid filtrates of the liver reaction mixtures by distillation in the presence of magnesium sulfate (10). Application of the following analyses showed that within the concentration ranges utilized a 95 to 100 per cent recovery of these acids was obtained.

Sorbic Acid—This acid was estimated directly in the distillate by its absorption at 255 mμ. The distillate was appropriately diluted to contain

from 0.002 to 0.025 μM of sorbic acid per ml. and was measured in quartz cells of 10 mm. light path in the Beckman spectrophotometer.

2-Δ-Hexenoic Acid—Analysis for this acid was based on the fact that, although 2-Δ-ethylenic fatty acids are resistant to ordinary bromination (42), they may be brominated in 50 per cent acetic acid (43). Within the concentration range and conditions chosen, 85 per cent of the theoretical amount of bromine reacts with the 2-Δ-hexenoic acid.

In practice, 10 ml. of distillate containing 1 to 5 μM of 2-Δ-hexenoic acid are placed in a 25 ml. glass-stoppered cylinder, followed by addition of 9 ml. of glacial acetic acid and 1.0 ml. of 0.006 M bromine in acetic acid. After mixing, the cylinder is placed in the dark at room temperature (25°) for 5 hours. 2 ml. of 5 per cent KI are then added, and the optical density of the liberated iodine is read at once at 430 mμ in the Beckman spectrophotometer. A standard curve with known amounts of 2-Δ-hexenoic acid is prepared for each set of analyses. Distillates of solutions containing triacetic or sorbic acids interfered with the method; acetoacetate, β-ketohexanoate, β-hydroxyhexanoate, β-hydroxybutyrate, hexanoate, or acetone did not interfere.

Results

Acetoacetate Formation from Hexanoate, 2-Δ-Hexenoate, and Sorbate at pH 8.0—In Tables I and II are illustrated the quantitative yields varying from 1.4 to 1.6 moles of acetoacetate per mole of hexanoate, 2-Δ-hexenoate, or sorbate, oxidized in both liver homogenate and washed liver particle systems. The same yield of acetoacetate per mole of fatty acid oxidized was obtained after partial or complete oxidation, indicating no extensive accumulation of intermediate products during the incubation.

In experiments with the washed liver system, addition of the heat-treated liver extract was necessary for rapid and complete oxidation of sorbate, but was without effect on the oxidation rate of the other two substrates. In other unrecorded experiments with rat liver mitochondria, a similar ratio of 1.5 moles of acetoacetate per mole of fatty acid oxidized was obtained, and with this material the heat-treated extract was needed for optimum oxidation of sorbate and 2-Δ-hexenoate but not for hexanoate. Lowering the pH of the washed liver reaction mixture to as low as pH 6.2 caused a progressively lower rate of production of acetoacetate from the fatty acids, but with the same ratio of acetoacetate production per mole of sorbate or 2-Δ-hexenoate oxidized. Estimation of hexanoate disappearance from oxygen consumption data was unreliable at the lower pH conditions owing to the relatively large oxygen consumption in the absence of added substrate. At the lower pH the oxidation of all three fatty acids was accelerated by addition of the heat-treated liver extract.

TABLE I

Acetoacetate Production in Rat Liver Homogenates

Reaction mixtures contained 0.02 M sodium phosphate buffer, pH 8.0, 0.01 M MgCl₂, 0.0025 M adenosine triphosphate, 2×10^{-6} M cytochrome c. Data recorded for a 2 ml. volume of reaction mixture containing 0.6 ml. of 33 per cent rat liver homogenate in modified Krebs-Ringer phosphate (44); in the case of hexanoic acid a 10 ml. reaction volume containing 3.0 ml. of homogenate was actually used. Temperature 30°; incubation 60 minutes.

Substrate	Substrate added	Substrate disappeared	Acetoacetate formed	$\dfrac{\text{Acetoacetate formed}}{\text{Substrate disappeared}}$
	μM	μM	μM	
Hexanoate.............	10.0	8.0	11.8	1.5
"	10.0	9.4	13.8	1.5
2-Δ-Hexenoate........	4.0	4.2	6.0	1.4
"	4.0	2.8	3.9	1.4
"	4.0	3.1	4.5	1.5
Sorbate................	3.0	3.0	4.5	1.5
"	3.0	2.1	3.0	1.4

TABLE II

Acetoacetate Production in Rat Washed Liver System

Reaction mixtures contained 0.01 M sodium phosphate buffer, pH 8.0, 0.01 M veronal buffer, pH 8.0, 6.6×10^{-3} M MgCl₂, 9×10^{-4} M adenosine triphosphate, 6.7×10^{-7} M cytochrome c, 0.03 M NaCl, 1×10^{-3} M substrate, and 0.4 ml. of washed liver particles in a total volume of 3.1 ml. In the case of sorbic acid, 0.6 ml. of heat-treated liver extract was added in place of 0.6 ml. of 0.85 per cent NaCl for optimum oxidation rate. Temperature 30°; reaction time shown.

Substrate	Incubation time	Substrate disappeared	Acetoacetate formed	$\dfrac{\text{Acetoacetate formed}}{\text{Substrate disappeared}}$
	min.	μM	μM	
Hexanoate.............	45	2.9	4.6	1.6
"	40	3.1	4.7	1.5
"	40	2.7	4.0	1.5
"	25	3.0	4.6	1.5
"	40	2.7	4.3	1.6
"	45	2.7	4.1	1.6
"	20	3.0	4.2	1.4
2-Δ-Hexenoate........	10	1.3	1.6	1.3
"	20	2.5	3.4	1.4
"	· 40	3.0	4.2	1.4
"	60	3.0	4.4	1.5
"	40	3.0	4.5	1.5
Sorbate................	20	1.0	1.5	1.5
"	40	2.6	3.9	1.5
"	60	2.7	4.0	1.5
"	60	3.0	4.6	1.5

It is clear from such quantitative experiments that the oxidation of these 6-carbon acids must involve the formation of 2-carbon fragments which can recondense to form acetoacetate. Since acetate does not form appreciable amounts of acetoacetate under the conditions of the experiments, acetate itself cannot be the 2-carbon fragment.

Effect of Inhibitors on Fatty Acid Oxidation—Several inhibitors were tested for their effect on the oxidation of hexanoate, 2-Δ-hexenoate, and sorbate. In the case of the unsaturated fatty acids both fatty acid disap-

TABLE III

Inhibitors of Acetoacetate Formation from 6-Carbon Fatty Acids in Rat Washed Liver System

Conditions same as those listed in Table II.

Inhibitor	Inhibitor concentration	Per cent of rate of acetoacetate formation without inhibitor		
		Hexanoate	Sorbate	2-Δ-Hexenoate
	$M \times 10^3$			
Benzoate..................	2.0	70	11	29
"	0.66	76	22	39
"	0.16	86	67	77
Phenylacetate.............	2.0	94	25	25
Cinnamate..............	0.66	27	0	26
"	0.12	56	25	41
Malonate................	10.0	91	70	37
"	3.0	96	85	56
Iodoacetate...............	1.3	67	56	47
"	1.3	71	43	33
Fluoride..................	2.0	25	33	23
"	0.66	60	60	60
Arsenite.................	1.3	15	16	11
"	0.66	50	30	30
Fluoroacetate............	10.0	100	100	96

pearance and acetoacetate formation were measured, from which it might be possible to detect the accumulation of intermediates due to the inhibitors. The data in Table III present only the effect of the inhibitors on the rate of acetoacetate formation from the three fatty acids.

In confirmation of previous work (6, 11, 45) the phenylated acids benzoate, phenylacetate, and cinnamate were found to be strong inhibitors of fatty acid oxidation. In addition to these compounds, malonate, arsenite, and iodoacetate were inhibitors and appeared to inhibit the oxidation of the unsaturated fatty acids more than hexanoate oxidation. In contrast to the reported fluoroacetate inhibition of fatty acid oxidation by washed rabbit kidney particles (11) and rat liver slices (46), no inhibition

was noted by 0.01 M fluoroacetate in the system here studied. Malonate was not a particularly effective inhibitor of hexanoate oxidation at pH 8.0, but at pH 6.2 to 7.0 was inhibitory. This variation with pH may be related to the discrepancies reported for this inhibitor (9, 11, 36, 45). 2,4-Dinitrophenol, brilliant cresyl blue, methylene blue, and ferricyanide, which "uncouple" the linkage between oxidation and phosphorylation (47–50), were also found to be potent inhibitors of fatty acid oxidation in the system employed.

TABLE IV

Acetoacetate Formation from Hexanoic Acid and Hydroxy, Keto, and Unsaturated Derivatives

A liver preparation washed once was used as enzyme source. 3 μM of the substrates were used. Other conditions of the experiments were the same as those outlined in Table II. Incubation time 60 minutes.

Substrate	Heat-treated extract added	Acetoacetate yield
		μM
Hexanoate	−	4.9
β-Hydroxyhexanoate	−	1.3
"	+	2.8
β-Ketohexanoate	−	0.5
"	+	1.1
2-Δ-Hexenoate	−	4.8
"	+	4.5
Sorbate	−	1.4
"	+	4.4
Triacetate	−	0
"	+	0
β-Hydroxy-γ-hexenoate	−	0.5
"	+	1.4
α,β-Dihydroxyhexanoate	−	0
"	+	0

In none of the combinations of fatty acids and inhibitors was there found an abnormal ratio of fatty acid disappearance to acetoacetate formation. Hence the inhibitor technique, within the limits used, did not provide a means of revealing the intermediates of fatty acid oxidation. Either the fatty acid did not disappear, or acetoacetate formation was the theoretical amount from that which did disappear.

Rates of Formation of Acetoacetate from 6-Carbon Fatty Acids—A true intermediate of hexanoate oxidation might be expected to yield the same product, acetoacetate, and possibly at a similar rate as hexanoate. Hence a number of 6-carbon acids were tested under identical conditions in the

washed rat liver system for their ability to form acetoacetate. Heat-treated liver extract was added to the test system with each substrate to attain the maximum rate of oxidation. The results are recorded in Table IV.

In the absence of heat-treated factor, only 2-Δ-hexenoate yielded acetoacetate at a rate comparable with hexanoate. Sorbate, β-hydroxyhexanoate, β-hydroxy-γ-hexenoate, and β-ketohexanoate gave low rates of acetoacetate production. The addition of heat-treated liver extract increased these rates, but only with sorbate did the rate approach that obtained from hexanoate or 2-Δ-hexenoate. Triacetate (β,δ-diketohexanoate) or α,β-dihydroxyhexanoate did not yield acetoacetate under the conditions tested.

In other experiments, decreasing the concentration of the substrates or

TABLE V

Adenosine Triphosphate and Oxygen Requirements for Sorbate and 2-Δ-Hexenoate Disappearance

Experimental conditions same as those outlined in Table II.

Substrate	Adenosine triphosphate	Air	Acid disappeared	Acetoacetic acid formed
			μM	μM
2-Δ-Hexenoate................	—	+	0.3	0.2
"	+	+	2.9	4.1
"	+	—	0.2	0.1
Sorbate......................	—	+	0.2	0.2
"	+	+	2.6	3.9
"	+	—	0.4	0.1

the time of incubation did not increase the rate of acetoacetate production relative to hexanoate. When it was possible to estimate disappearance of the substrate by direct analysis, all that which disappeared was accounted for by acetoacetate formation. β-Hydroxybutyrate, acetaldehyde, or citrate could not be detected as products of the reaction.

Sorbate and 2-Δ-Hexenoate Oxidation—Since these two unsaturated acids were rapidly oxidized in the appropriately fortified liver preparation, and methods were available for their determination, they were further studied with respect to requirements for their oxidation and possible reaction products. Table V illustrates the requirement of adenosine triphosphate and oxygen for disappearance of these acids. Muscle adenylic acid can replace adenosine triphosphate.

It may be noted that oxygen is necessary for the disappearance of the unsaturated acids, a fact which does not support a mechanism of simple addition of water to these acids. The theoretical product of water addi-

tion to 2-Δ-hexenoate, namely β-hydroxyhexanoate, did not inhibit the aerobic disappearance of the unsaturated acid when added even at double the concentration of the 2-Δ-hexenoate. The failure of α-β-dihydroxyhexanoate to be oxidized (Table IV) does not support a rôle of this compound in the oxidation of 2-Δ-hexenoate.

Heat-Treated Factor—The nature of the active compound or compounds in the heat-treated liver extract is unknown. Since the activity was destroyed on ashing, the compound appears to be organic. With sorbate, the oxidation of which was greatly accelerated by the heat-treated factor, the action of the factor was not duplicated by HCO_3^-, NH_4^+, coenzyme A, glucose, muscle adenylic acid, or adenosine triphosphate. A small amount of a dicarboxylic acid, such as α-ketoglutarate, succinate, fumarate, or *l*-malate, simulated the action of the factor, but an excess of dicarboxylic acid acted differently than a corresponding excess of factor. Thus the addition of 0.35 μM of α-ketoglutarate to the usual washed liver system caused an optimum rate of acetoacetate formation and a yield of 1.5 moles per mole of sorbate disappeared, while 1.1 μM of α-ketoglutarate reduced acetoacetate formation and decreased the yield to about 1.0 mole per mole of sorbate oxidized. In contrast, a 3-fold excess of the amount of heat-treated factor which caused an optimum acetoacetate formation caused no change in the rate or yield of acetoacetate from sorbate. Under conditions of a lowered yield of acetoacetate no β-hydroxybutyrate was detected; hence, it seems likely that the decreased yield of acetoacetate is due to oxidation of a part of the sorbate through the Krebs cycle, as was found in the case of octanoate oxidation by Lehninger and Kennedy (45).

DISCUSSION

The formation of 1.5 moles of acetoacetate for each mole of hexanoate, 2-Δ-hexenoate, or sorbate, which disappears in a liver enzyme system incapable of metabolizing either acetate or acetoacetate, is direct evidence for a β-oxidation-recondensation mechanism for the oxidation of these 6-carbon acids. In earlier studies of hexanoate oxidation by liver slices (6, 7) a lower yield of acetoacetate was interpreted as supporting a mechanism of multiple alternate oxidation with cleavage to acetoacetate and acetate. These and other (12, 46, 51) studies either suffered from difficult analytical estimation of fatty acid disappearance, further metabolism of acetate or acetoacetate in the system employed, or other factors which made exact determination of substrate disappearance or end-product formation difficult.

The data in support of the recondensation mechanism reported in this paper are consistent either with splitting of the 6-carbon acids to three 2-carbon fragments or to acetoacetate and one 2-carbon fragment. In

view of the recondensation mechanism demonstrated for acetoacetate formation from isotopically labeled butyrate (2), the former mechanism seems most likely for hexanoate metabolism. This mechanism seems to be at variance however with the *in vivo* experiments of Morehouse and Deuel (8) which appear to support a multiple alternate oxidation pathway for the metabolism of deutero-labeled hexanoate.

Some information about the nature of the intermediates of hexanoate oxidation might be gained by a comparison of rates of acetoacetate production from postulated intermediates and the parent substance hexanoate. There are difficulties, however, in the interpretation of such data. The comparable rates of hexanoate, 2-Δ-hexenoate, and sorbate oxidation argue for dehydrogenation of the fatty acid as a first step in metabolism. The failure of the unsaturated acids to disappear anaerobically and the relatively slow rate of disappearance of β-hydroxyhexanoate and β-ketohexanoate do not support simple addition of water to 2-Δ-hexenoic acid and subsequent oxidation. In the case of crotonic acid oxidation, Jowett and Quastel (6, 52, 53) and Lipmann and Perlmann (54) came to a similar conclusion. These observations indicate that the hydroxy and keto acids named are not *per se* intermediates of hexanoate oxidation, but, in view of the adenine nucleotide requirements of fatty acid oxidation, it is not improbable that phosphorylated derivatives are actually involved. In this case, rate studies involving the non-phosphorylated compounds may not be valid in determining the intermediates of fatty acid oxidation.

The complete failure of oxidation in the case of α,β-dihydroxyhexanoate and β,δ-diketohexanoate (triacetate) certainly does not favor these compounds as obligatory intermediates of hexanoate oxidation in the washed liver system employed. In the case of the 4-carbon acids, Krebs and Eggleston (55) and Weil-Malherbe (56), using minced sheep heart or kidney cortex, found that α,β-dihydroxybutyrate did not form acetoacetate at a rapid enough rate to be considered an intermediate in the oxidation of butyrate or crotonate. The occurrence of a soluble liver enzyme which hydrolyzes triacetate to acetoacetate and acetate (57) indicates that whole liver tissue may display phases of fatty acid oxidation or synthesis which are not evident in the washed insoluble fraction of liver. In the experiments of Grafflin and Green (11), who used a somewhat different enzyme system of rabbit liver, similar relative rates of oxidation of several postulated intermediates were found as are recorded in this paper for the optimum oxidation of these intermediates attained in the presence of the heat-treated factor.

Although several inhibitors of fatty acid oxidation were found, it was not found possible to dissociate fatty acid disappearance and acetoacetate formation and thus to permit accumulation of intermediates.

Lipmann and Perlmann (54) have suggested that during fat oxidation phosphoric acid adds to a double bond producing a high energy bond, which mechanism might account for the necessity of adenine nucleotide and aerobic conditions in the metabolism of the unsaturated acids. Knox and coworkers (48) were unable to detect acyl phosphates during fatty acid oxidation in a rabbit-washed particle system. More work on phosphorylation during fatty acid oxidation is clearly required.

SUMMARY

Quantitative measurements of acetoacetate production from various 6-carbon acids were made with rat liver homogenates and washed liver particulate matter, for which analytical methods for 2-Δ-hexenoate and sorbate were developed. A yield of 1.5 moles of acetoacetate from 1 mole of hexanoate, 2-Δ-hexenoate, or sorbate proved that a recondensation mechanism was involved. Rates of acetoacetate formation from possible intermediates of hexanoate oxidation showed that only the unsaturated acids 2-Δ-hexenoate and sorbate gave rates comparable to hexanoate in the washed liver system. β-Hydroxy and β-ketohexanoate were more slowly oxidized, and α,β-dihydroxy hexanoate and triacetate were not affected. Disappearance of the unsaturated acids was dependent on oxygen and the presence of adenine nucleotide. The use of inhibitors did not reveal accumulation of fatty acid oxidation intermediates. The significance of the results to the mechanism of fatty acid oxidation is discussed.

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ACETYL PHOSPHATE SYNTHESIS BY REACTION OF ISOPROPENYL ACETATE AND PHOSPHORIC ACID

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Isopropenyl acetate has been shown to be a particularly useful reagent for the acetylation of organic compounds when mild acetylation conditions are required (1). The use of this cheap reagent for the acetylation of phosphoric acid is the basis of a new simplified procedure for the synthesis of monoacetyl dihydrogen phosphate.

$$CH_2{=}\underset{\underset{\displaystyle CH_3}{|}}{C}{-}O{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}CH_3 + H_3PO_4 \longrightarrow CH_3COOPO_3H_2 + (CH_3)_2CO$$

The present reaction was suggested by the previous synthesis involving the reaction of ketene and phosphoric acid (2).

Procedure

200 ml. of isopropenyl acetate (Tennessee Eastman Corporation) are cooled in an ice bath and then, with stirring, 25 ml. of 85 per cent syrupy phosphoric acid (35 gm. of H_3PO_4) are added dropwise, and finally 1.0 ml. of concentrated sulfuric acid is added as catalyst. The reaction mixture is placed in a water bath at 25° and stirred constantly to prevent localized heating. After 35 minutes at this temperature, the reaction mixture becomes deep orange in color. It is then cooled in an ice bath and 50 ml. of ice-cold water are added cautiously. A too rapid addition of water results in a rapid rise in temperature and consequently lower yields of acetyl phosphate.

The mixture is then neutralized to about pH 5.0 with cold 4 N lithium hydroxide (about 300 ml.). During the neutralization, the mixture may separate into two phases, since the isopropenyl acetate which was added in excess is relatively insoluble in water. It is therefore necessary to mix the layers thoroughly while adding the lithium hydroxide so as to extract the acetyl phosphate from the organic phase. The neutralization should be carried out as rapidly as possible without, however, causing the tem-

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perature to rise unduly. Once the pH is adjusted to 5.0, the acetyl phosphate is relatively stable (3) and subsequent operations may be carried out more leisurely.

Finally, enough cold water is added to bring the aqueous solution to about 500 ml. in volume and the mixture is extracted three times with 500 ml. amounts of ethyl ether to remove the excess isopropenyl acetate. The aqueous portion is then adjusted to pH 8.0 with 4 N lithium hydroxide and is centrifuged to remove the lithium phosphate.

The supernatant is placed in an ice bath and, with stirring, 5 volumes of cold ethyl alcohol are added. The dilithium monoacetyl phosphate is precipitated (4) and filtered, washed with absolute ethanol and finally with ether, and is then dried in a vacuum desiccator over phosphorus pentoxide and paraffin.

By the above procedure, 40 to 45 gm. of dry material were obtained. This crude product contains about 80 per cent dilithium monoacetyl phosphate. The calculated yield is therefore 60 to 70 per cent. The synthesis has been carried out on a smaller scale (one-twentieth of the above) with comparable yields.

The crude product is purified by fractional precipitation from a 10 per cent solution by addition of ethanol. The precipitate formed with 0.5 volume of ethanol contains most of the impurities and is discarded. The compound is precipitated by further slow addition of 5 volumes of cold ethanol. After two such reprecipitations, a relatively pure compound is obtained.

$C_2H_4O_5PLi_2$ Calculated.   Acetic acid 39.5, P 20.4
151.8   Found.   "   "   37,   " 20.3

Acetic acid was determined by steam distillation from the acidified solution and was titrated.

Such a preparation is very suitable for most biological work. For rigorous purification, a conversion into the easily crystallized silver salt has been applied and an analytically pure preparation was obtained.

The compound was further identified as acetyl phosphate by reaction with hydroxylamine to give a hydroxamic acid under the conditions of Lipmann and Tuttle (5). It was also found to be biologically active in enzyme preparation of *Clostridium kluyveri*, which were shown previously (6) to utilize monoacetyl phosphate.

SUMMARY

A new convenient method is described for the synthesis of monoacetyl phosphate by reaction of isopropenyl acetate and phosphoric acid. Sul-

furic acid is added as a catalyst. Acetylphosphoric acid, obtained in goo
yield, is isolated and purified as the dilithium salt.

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ANTAGONISMS IN THE UTILIZATION OF D-AMINO ACIDS BY LACTIC ACID BACTERIA

II. INFLUENCE OF DL-SERINE AND GLYCINE ON THE UTILIZATION OF D-ALANINE*

BY MERRILL N. CAMIEN AND MAX S. DUNN

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The authors have shown previously that the utilization of D-glutamic acid (1) and D-methionine (2) by *Lactobacillus arabinosus* is strongly inhibited by relatively high concentrations of aspartic acid and ethionine, respectively. Other workers (3, 4) have found that leucine and valine in moderate concentrations may interfere with the utilization of D-isoleucine by *L. arabinosus*. On the basis of these findings the authors have hypothecated that "the greater reduction by specific amino acid inhibitors of D-amino acid activity than of L-amino acid activity for lactic acid bacteria probably is a general phenomenon" (2). Experiments designed to test this hypothesis with D-alanine utilization by lactic acid bacteria as influenced by increased glycine and serine concentrations are described in the present report.

EXPERIMENTAL

Medium—A dry mixture containing 1 gm. each of L-arginine monohydrochloride, L-asparagine monohydrate, L-cysteine hydrochloride, L-glutamic acid, L-histidine monohydrochloride monohydrate, DL-isoleucine, L-leucine, L-lysine monohydrochloride, DL-methionine, DL-phenylalanine, L-proline, DL-threonine, DL-tryptophan, L-tyrosine, and DL-valine was pulverized in a glass mortar. 900 mg. of the amino acid mixture, 5.16 gm. of vitamin-salt mixture B (given in Table I of Paper 65 (5)), 6 gm. of glucose, and 60 mg. each of glycine and DL-serine were dissolved in distilled water[1] and diluted to 200 ml. to prepare basal medium for 100 assay tubes. The basal medium was modified by increasing and decreasing the concentrations of glycine and DL-serine and by introducing L- and D-alanine to study the effects of these amino acids. In some of the experiments with *Leuconostoc*

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[1] A small amount of insoluble material remains and may be removed by decantation.

citrovorum (8081)[2] the basal medium was further modified by replacing the amino acid mixture given above with that from Medium VI of Sauberlich and Baumann (6). All media for use with *L. citrovorum* were supplemented with liver concentrate[3] either reticulogen as recommended by Steele *et al.* (7), or the preparation described previously (2). 2 ml. of medium were added to 1 ml. of test sample in each 13 × 100 mm. test-tube.

The cultures of *Lactobacillus lycopersici* (4005),[2] *Lactobacillus mannitopoeus, Leuconostoc mesenteroides* P-60 (8042), *L. citrovorum* (8081), and *Streptococcus faecalis* R (8043) were the same as those described previously (8). The inocula were prepared and introduced as described in Paper 47 (9). The response of each organism to graded amounts of L-, DL-, and D-alanine was determined titrimetrically[4] after incubating for 72 hours (except as otherwise stipulated) at 35°.

The results are given in Tables I to III and in Figs. 1 to 5.

<center>DISCUSSION</center>

The effects of glycine and serine on L-, DL-, and D-alanine utilization were studied because the former amino acids have been shown to be inhibitory under certain conditions for *S. faecalis* (10) and *Escherichia coli* (11) with reversal of the inhibition by alanine. That glycine and serine may compete with alanine in living systems involving this amino acid would be expected from the close structural similarity of the three compounds.

Increases in glycine and serine concentrations were invariably accompanied by corresponding decreases in the relative activity of D-alanine (compared with L-alanine) in the present experiments (Tables I to III, Figs. 1 to 3). The converse of this was also true except for *L. mesenteroides* which showed no change in relative D-alanine activity at lowered glycine and serine concentrations and for *L. mannitopoeus* which showed a decrease in relative D-alanine activity under these conditions (Table I). Such ex ception was not unexpected for *L. mesenteroides*, since this bacterium appeared to be unable to utilize D-alanine under any of the conditions tested.

It is of interest that *S. faecalis* showed no response to D-alanine in the unmodified basal medium, but was able to utilize this compound partially at lowered glycine and serine concentrations (Table I). It also seems note-

[2] The numbers in the parentheses are catalogue numbers of the American Type Culture Collection.

[3] No differences in results were found with the different liver preparations.

[4] Growth was terminated by steaming the cultures 20 minutes at 100°. The contents of triplicate tubes (total of 9 ml.) were combined in 50 ml. conical flasks with 3 drops of indicator (0.8 per cent brom thymol blue in 50 per cent ethanol) and titrated with 0.09 N NaOH. The advantages of this titration procedure have been discussed previously (9).

worthy that DL-serine was considerably more effective than glycine in reducing the relative activity of D-alanine for *L. citrovorum*, although the two compounds were nearly equally effective as inhibitors of the response to L-alanine by this bacterium (Table II).

The sequence of effects produced by graded increases in glycine concentration on the response of *L. mannitopoeus* to L-, DL-, and D-alanine is illus-

TABLE I

Influence of Glycine and DL-Serine on Relative Activity of D-Alanine for Five Bacteria

Organism	Activity of D-alanine		
	Medium 1	Medium 2	Medium 3
	per cent	*per cent*	*per cent*
L. lycopersici.............	41 (2.7)	72 (2.1)	8* (8.3)
L. mannitopoeus†........	208 (23)	125 (22)	149 (44)
L. citrovorum.............	40 (12)	66 (9.3)	35‡ (27)
L. mesenteroides..........	2* (47)	2* (43)	§
S. faecalis...............	4* (25)	20 (8.0)	0* (270)

Medium 1 was the basal medium (given in the text). *Medium 2* was the basal medium modified to contain one-fourth the original glycine and DL-serine concentrations. *Medium 3* was the basal medium modified to contain 30 times the original glycine concentration.

The D-alanine activity was calculated as follows (except as otherwise stipulated): per cent activity =

$$\frac{100 \times \text{concentration of L-alanine allowing half maximum acid production}}{\text{concentration of D-alanine allowing half maximum acid production}}$$
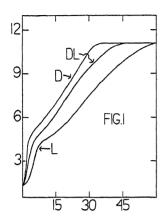
The values given in the parentheses are the concentrations in micrograms per ml. of L-alanine required to allow half maximum acid production.

* Half maximum acid was not produced at the highest D-alanine concentration tested. The value given was calculated from the data yielding the highest apparent activity.

† Incubated 40 hours. All others were incubated 72 hours. The shorter incubation period was employed for *L. mannitopoeus*, since this organism is able to grow in the absence of alanine at longer incubation times (8).

‡ Medium contained 500 mg. per cent of glycine.

§ *L. mesenteroides* does not require alanine in Medium 3.

trated in Figs. 1 to 3. It may be seen (Fig. 1) that in the unaltered basal medium D-alanine was somewhat more active than DL- and L-alanine. A 30-fold increase in the glycine concentration rendered D-alanine less active, at high dosage levels, than DL- and L-alanine (Fig. 2), and a 120-fold increase in the glycine concentration (Fig. 3) markedly reduced the activity of both L- and D-alanine relative to that of DL-alanine.

In effect, at the highest glycine concentration (Fig. 3), L-alanine and D-alanine had become independent nutrient essentials for *L. mannitopoeus*,

This fact was emphasized by additional experiments in which response to L-alanine but not to D-alanine was obtained in the high glycine medium to
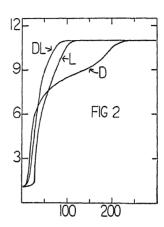
FIG. 1. Response of *L. mannitopoeus* to L-, DL-, and D-alanine with 40 hours incubation (see dagger foot-note, Table I) in the basal medium (given in the text). The values on the horizontal scale represent the concentration of alanine in micrograms per ml. The values on the vertical scale represent the ml. of 0.01 N NaOH required to titrate 1 ml. of culture.

FIG. 2. Same as Fig. 1 except that the concentration of glycine in the basal medium was increased 30 times.

<div align="center">TABLE II</div>

Relative Influence of Glycine and DL-Serine on Utilization of L-, DL-, and D-Alanine by L. citrovorum

Alanine	Alanine concentration at half maximum acid production		
	No addition	Glycine	DL-Serine
	γ per ml.	γ per ml.	γ per ml.
L-	11	27	28
DL-	15	36	50
D-	18	76	150

The basal medium was that with the amino acid mixture of Sauberlich and Baumann (6) (see the text). The additions of glycine and DL-serine were in amounts giving final concentrations of 500 mg. per cent.

which D-alanine had been added (Fig. 4), and response to D-alanine but not to L-alanine was obtained in the high glycine medium to which L-alanine had been added (Fig. 5). These experiments appear to be the first in
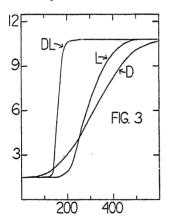
which both enantiomorphs of an amino acid have been shown to be simultaneously essential in bacterial nutrition.

TABLE III

Response of L. citrovorum to L-, DL-, *and* D-*Alanine with Varying Concentrations of* DL-*Serine*

DL-Serine concentration	Alanine concentration at half maximum acid production		
	L-Alanine	DL-Alanine	D-Alanine
mg. per cent	γ *per ml.*	γ *per ml.*	γ *per ml.*
0	11	13 (85)	16 (69)
200	17	31 (55)	93 (18)
400	23	43 (53)	145 (16)
600	39	76 (51)	293 (13)
800	40	69 (58)	320 (12)
1000	47	77 (61)	447 (11)

The basal medium was that with the amino acid mixture of Sauberlich and Baumann (6) (see the text). The given DL-serine concentrations were in addition to that already present in the basal medium. The values in parentheses are the activities in per cent calculated as in Table I.
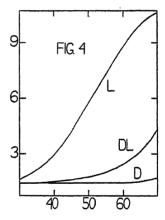
FIG. 3. Same as Fig. 1 except that the concentration of glycine in the basal medium was increased 120 times.

FIG. 4. Same as Fig. 3 except that the medium contained 16.7 mg. per cent (final concentration) of D-alanine.

It is apparent that the separate microbiological determination of L- and D-alanine may be possible under conditions similar to those described for *L. mannitopoeus* in the above experiments. The development of such assay methods is in progress in the authors' laboratory.

It is of interest that with *L. citrovorum* in the present experiments the highest activity found for D-alanine was only 66 per cent of that found for L-alanine (Table I). Sauberlich and Baumann (12) have reported that both DL- and D-alanine were approximately as active for *L. citrovorum* as was L-alanine. The data given by these authors in their Table II (12) indicate, however, that even in their experiments the average activity of D-alanine was only about 86 per cent, compared with L-alanine. This possibly variable response of *L. citrovorum* to D-alanine should probably be considered as a potential source of error in alanine assays with this organism of samples which might contain D-alanine.

The results of the present experiments appear, in general, to support the
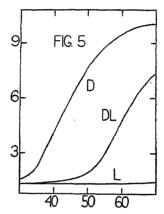
FIG. 5. Same as Fig. 3 except that the medium contained 16.7 mg. per cent (final concentration) of L-alanine.

hypothesis that "the greater reduction by specific amino acid inhibitors of D-amino acid activity than of L-amino acid activity for lactic acid bacteria probably is a general phenomenon" (2). This hypothesis would not necessarily be expected to hold true, however, in those instances (like that of *L. mannitopoeus* in the present experiments) in which both enantiomorphs of the test amino acid are essential metabolites for the test organism, because the reaction yielding the D-amino acid from the L form could be blocked by the inhibitor as well as the reverse reaction, and there appears to be no reason to believe that the reverse reaction should be blocked more effectively than the other in all cases. It might be anticipated, though, that both reactions (L-amino acid → D-amino acid, and D-amino acid → L-amino acid) would be blocked by sufficiently high levels of inhibitor in nearly all cases, thus providing a new means of demonstrating the essential character of D-amino acids in the nutrition of bacteria.

Another means which has been employed successfully to inhibit the interconversion of L- and D-amino acids in bacterial nutrition is that of employing media deficient in vitamin B₆, which appears to be essential for certain of these interconversions.[5] By this method the authors have suppressed the utilization of D-methionine by L. arabinosus (13), and Holden, Furman, and Snell (14, 15) have demonstrated the essential character of D-alanine for various lactic acid bacteria.

A third method which suggests itself for avoiding interconversion of L- and D-amino acids in bacterial nutrition is that of producing biochemical mutants deficient in the enzyme systems employed in the interconversions. All three of these methods are now being employed in the authors' laboratory to study D-amino acids in the nutrition of bacteria. Details of these investigations will be given in the papers to follow in this series.

SUMMARY

The utilization of L-, DL-, and D-alanine by lactic acid bacteria as affected by the inhibitors, glycine and serine, has been studied. It was shown that the interconversion of L- and D-amino acids in bacterial nutrition may be suppressed by these inhibitors, and by this means D-alanine was demonstrated to be an essential metabolite for L. mannitopoeus. A potential microbiological assay procedure for the separate determination of L- and D-alanine was suggested, and other implications of these findings were discussed.

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[5] Sauberlich and Baumann (12) have reported that the presence or absence of vitamin B₆ does not affect the relative activity of D-alanine for L. citrovorum.

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STUDIES ON THE MUCOPROTEINS OF HUMAN PLASMA

V. ISOLATION AND CHARACTERIZATION OF A HOMOGENEOUS MUCOPROTEIN*

By HENRY E. WEIMER, JOHN W. MEHL, AND RICHARD J. WINZLER

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Previous publications in this series have dealt with the determination and isolation of plasma mucoproteins (1), their increase in cancer and certain other pathological conditions (2), electrophoretic studies of plasma mucoproteins isolated from perchloric acid filtrates of plasma (3), and the direct demonstration of mucoproteins in normal and pathological sera by electrophoresis (4). Mucoproteins characterized in the above communications were found to be a mixture of at least three components (1, 3). A preparation obtained by ammonium sulfate fractionation (3) was more nearly homogeneous electrophoretically than those obtained from perchloric acid filtrates. It has now been possible to isolate the major component of normal plasma mucoprotein in an electrophoretically homogeneous state by a series of ammonium sulfate precipitations. This mucoprotein was designated as MP-1 in a previous paper (3), and appears to be identical with mucoprotein M-1 in circulating plasma (4).

The present work deals with the isolation and chemical and electrophoretic characterization of this mucoprotein.

Isolation

The method employed for the isolation of the major plasma mucoprotein component is outlined in the accompanying diagram.
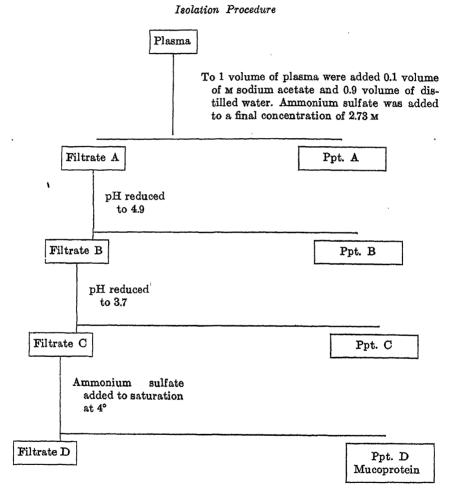
1 volume, usually 1 to 3 liters, of pooled, normal human plasma[1] was diluted with 0.1 volume of 1 M sodium acetate and 0.9 volume of distilled water. Ammonium sulfate was added with stirring to a concentration of

2.73 M. The mixture was allowed to stand for a minimum of 16 hours at 4° and then was filtered through a double thickness of Whatman No. 1 filter paper. The pH of the filtrate was reduced to 4.9 with 1 N hydrochloric acid, and the resulting suspension was allowed to stand at 4° for a

Isolation Procedure

Plasma

To 1 volume of plasma were added 0.1 volume of M sodium acetate and 0.9 volume of distilled water. Ammonium sulfate was added to a final concentration of 2.73 M

Filtrate A   Ppt. A

pH reduced to 4.9

Filtrate B   Ppt. B

pH reduced to 3.7

Filtrate C   Ppt. C

Ammonium sulfate added to saturation at 4°

Filtrate D   Ppt. D
Mucoprotein

minimum of 16 hours. The precipitate was removed by filtration through a double thickness of Whatman No. 1 filter paper as before. The pH of the filtrate was then reduced to 3.7 with 1 N hydrochloric acid, the mixture was again allowed to stand at 4° for a minimum of 16 hours, and the precipitated proteins were filtered as above. The mucoprotein was then

precipitated by saturating the filtrate with ammonium sulfate at 4°. The mixture was allowed to stand for a minimum of 72 hours at 4° to insure complete precipitation of the mucoprotein which was collected by filtration through a double thickness of Whatman No. 1 filter paper. It was then dissolved in a small volume of water, dialyzed against distilled water until free of ammonium sulfate, and dried by lyophilization.

The preparations were then dissolved to a concentration of 2 per cent, filtered, and reprecipitated at pH 3.7 with saturated ammonium sulfate and again isolated. This procedure has consistently yielded 500 ± 50 mg. of an electrophoretically homogeneous mucoprotein per liter of plasma when volumes of plasma up to 3 liters were fractionated. The fractionation of larger volumes has yielded a slightly inhomogeneous product which could be rendered homogeneous by refractionating.

The somewhat hygroscopic preparations are readily soluble in water to give faintly turbid solutions which do not yield a coagulum even when acidified with acetic acid and boiled. The mucoprotein is not precipitated by 0.6 M perchloric acid, by 0.2 M sulfosalicylic acid, or by 5 per cent trichloroacetic acid, but is precipitated by phosphotungstic acid and by ethyl alcohol at 75 per cent concentration at pH 3.5. The relative viscosity of 2 per cent aqueous solution of the mucoprotein was 1.165.

Chemical Composition

The chemical composition of the isolated protein given in Table I shows the characteristically high carbohydrate and hexosamine and low nitrogen content previously noted and thus would fall into the category of mucoproteins according to the classification of Meyer (10) and of Stacey (11).

It was thought that the high hexosamine content might only be apparent, having arisen from the reaction of carbohydrate with amino acids. However, no positive test for this reaction was obtained by the procedure of Vasseur and Immers (12).

It was suspected that some insight into the source of the plasma mucoprotein might be obtained from an examination of its amino acid composition. Accordingly the protein was subjected to amino acid analysis by microbiological assays.[2] The content of amino acids shown in Column 2 of Table II does not correspond to that of any of the plasma proteins studied in detail (13). The differences between the amino acid composition of MP-1 and serum albumin become less significant, however, when the amino acid content of MP-1 is multiplied by the factor 105.9/58.24 to compare the amounts of protein (Columns 3 and 4). This factor is the ratio of the per cent of the known amino acids in plasma albumin (105.9) and MP-1 (58.2). However, significant differences between the

[2] Performed by Shankman Laboratories, Los Angeles, California.

TABLE I

Chemical Composition of Normal Human Plasma Mucoprotein (MP-1)

Component	Method	Amount
		*gm. per 100 gm.**
Hexose.............	Orcinol reaction (as galactose-mannose (5)) †	16.4
Hexosamine........	Acetylacetone (as glucosamine (6))	11.9
Nitrogen...........	Digestion, aeration, titration (7)	10.1
Amide nitrogen.....	Micro diffusion	0.7
Lipide..............	Hot alcohol extraction	3.6
Ash................	Ignition in muffle furnace	1.8
Sulfur..............	Gravimetric	1.02
Phosphorus.........	Fiske-Subbarow (8)	0.03
Hexuronic acid.....	Carbazole (9)	Negligible

* Moisture-free basis.
† The figures in parentheses refer to the bibliography.

TABLE II

Amino Acid Composition of Human Plasma Mucoprotein (MP-1) and Human Plasma Albumin (Edsall (13))

Component (1)	Mucoprotein (2)	Mucoprotein (3)	Albumin (4)
	*gm. per 100 gm.**	*gm. per 182 gm.*	*gm. per 100 gm.*
Arginine...............................	3.65	6.7	6.2
Aspartic acid..........................	7.44	13.5	10.4
Cystine and Cysteine..................	0.60	1.1	5.6
Glycine................................	0.82	1.5	1.6
Glutamic acid..........................	10.73	19.6	17.4
Histidine..............................	1.31	2.4	3.5
Isoleucine.............................	3.15	5.7	1.7
Leucine................................	5.21	9.5	11.0
Lysine.................................	5.03	9.2	12.3
Methionine............................	0.65	1.2	1.3
Phenylalanine..........................	3.91	7.1	7.8
Proline................................	2.37	4.3	5.1
Threonine.............................	4.80	8.7	5.0
Tryptophan............................	1.25	2.3	0.2
Tyrosine...............................	1.99	3.6	4.7
Serine.................................	2.51	4.6	3.7
Valine.................................	2.82	5.1	7.7
Total................................	58.24	106.1	105.9

* Gm. of amino acid liberated by hydrolysis of 100 gm. of MP-1, moisture-free, ash-free basis.

proteins persist, especially for the amino acids cystine, isoleucine, and tryptophan. It seems unlikely that serum albumin is a direct precursor of MP-1. The cystine and methionine values account for 0.22 per cent of the sulfur in comparison to the 1.02 per cent actually found. This may be explained by the presence of sulfuric acid esters which would also account for the low isoelectric point exhibited by the mucoprotein. This possibility was supported by the observation that, after mild acid hydrolysis of the mucoprotein, a precipitate was produced by the addition of barium chloride or benzidine in acetic acid.
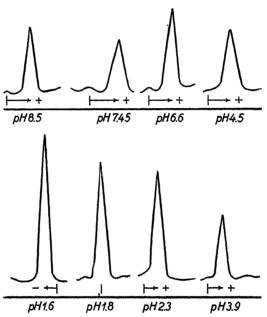
Fig. 1. Electrophoretic patterns of mucoprotein (MP-1) at various pH values. The vertical lines below each pattern indicate the position of the starting boundary.

The nitrogen contained in the amino acids, shown in Table II, together with that of the hexosamine and the amide, shown in Table I, accounts for 90 per cent of the nitrogen actually found in MP-1. This value does not include any alanine which may be present. Only 86 per cent of total weight of MP-1 is accounted for by the indicated amino acids (corrected for water of hydrolysis), hexose, hexosamine, lipide, ash, sulfur, and amide nitrogen.

Electrophoretic Studies

The mucoprotein was found to be electrophoretically homogeneous at pH values from 1.5 to 8.5. The electrophoretic studies were carried out

in a modified Tiselius apparatus by the method of Longsworth (14). Bar-biturate, acetate, trichloroacetate, phosphate, and perchlorate buffers were employed. The buffer systems were 0.02 M with respect to the so-dium or potassium salts of the buffer anion and 0.08 M in sodium chloride. The desired pH was obtained by adding hydrochloric acid; so that the final ionic strength was 0.1 except in the case of the phosphate buffer sys-
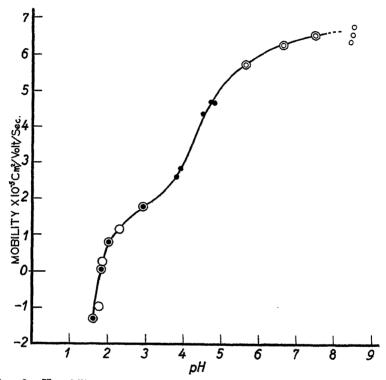
FIG. 2. pH-mobility curve of mucoprotein (MP-1). Barbiturate buffer (O), phosphate buffer (◉), acetate buffer (●), trichloroacetate buffer (◉), perchlorate buffer (O).

tem. 1 per cent solutions of mucoprotein in buffer were prepared and were dialyzed for 48 hours against two changes of buffer. The mucopro-tein solution was centrifuged if necessary to clarify it prior to filling the cell. Electrophoresis was carried out at 4 to 6 volts per cm. for 3 to 6 hours at a temperature of 2°. Mobilities were computed from the descending boundary.

Fig. 1 shows the descending boundaries after electrophoresis at the in-dicated pH values. These patterns demonstrate that the mucoprotein

isolated by this procedure appears electrophoretically homogeneous over the entire pH range studied. This is, of course, not an absolute criterion of the purity of the protein, since small amounts of impurities are not detected by this method. However, the homogeneity of the material over the wide range of pH values used in this investigation limits the possibility that the preparations contain more than one major component.

Fig. 2 shows the pH-mobility curve obtained by plotting the mobility of the descending boundary against the pH of the solution. The isoelectric point of the mucoprotein is at pH 1.8. This is an unexpectedly low value for a protein occurring in plasma.

DISCUSSION

The mobilities and chemical composition of the electrophoretically homogeneous protein described in thi work correspond to the major component present in the perchloric acid filtrates of normal human plasma which has been designated as MP-1 (3) and also to the fastest moving component negatively charged at pH 4.5 in normal or pathological serum designated as M-1 (4). The concentration of mucoprotein rises sharply in malignant disease as well as in such conditions as tuberculosis and pneumonia (2). The frequently observed increase in the α_1-globulin fraction in the plasma of patients with these conditions is in part associated with the fact that the mucoprotein MP-1 migrates with the α_1-globulin at pH 8.6 in the barbiturate buffer usually employed for examination of serum or plasma. This was shown in a previous paper (4) and has again been verified with the homogeneous mucoprotein obtained in this work.

The low isoelectric point of MP-1 is most likely due to its content of sulfuric acid esters, possibly in combination with carbohydrate components. From the molecular weight of 44,100 (15) it is apparent that there are 14 or 15 atoms of sulfur per molecule. Only 4 are accounted for by the cystine and methionine content, leaving 10 or 11 sulfur atoms present in another form, some of which have been demonstrated to be present as sulfate.

It is to be noted that the isoelectric point of 1.8 obtained with the present preparations is somewhat lower than the value of 2.3 observed (3) for the MP-1 previously isolated from perchloric acid filtrates of plasma. This may be due to the difference between the buffer systems employed in this and the previous study.

The present study minimizes the possibility that the increased plasma mucoprotein associated with cancer or infectious diseases arises from the degradation of plasma proteins as suggested by Brdicka et al. (16). Although the amino acid composition does not preclude the possibility that the protein portion of the mucoprotein is related to some other plasma

protein, the high carbohydrate and probable high sulfuric acid ester content cannot be reconciled with an origin by simple degradation of other plasma proteins.

Preliminary studies indicate that this mucoprotein (MP-1) is similar to the seromucoid of earlier workers in that both proteins have a relatively high hexose and hexosamine content and a relatively low nitrogen content in comparison with other plasma proteins, and are not coagulated by heat. Electrophoretically, MP-1 and seromucoid have been found to differ in the number of components, in the mobilities, and in isoelectric points. The comparison of MP-1 with the carbohydrate-rich protein fractions isolated from serum by other workers will be the subject of a future communication.

SUMMARY

A method has been described for the isolation of an electrophoretically homogeneous mucoprotein from human plasma. Material so obtained has been characterized chemically and electrophoretically. The preparation has a relatively high carbohydrate and hexosamine content and relatively low nitrogen content in comparison with other plasma proteins. The amino acid composition resembles that of other plasma proteins but differs significantly from albumin in certain amino acids. The mucoprotein was found to have its isoelectric point at pH 1.8.

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SEDIMENTATION, DIFFUSION, AND MOLECULAR WEIGHT OF A MUCOPROTEIN FROM HUMAN PLASMA*

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Investigations by some of us (1–3) have been concerned with the demonstration by electrophoresis of mucoproteins in normal and pathological sera. It was found that at least three electrophoretic components were present in crude mucoprotein fractions. More recently, the isolation in electrophoretically homogeneous form of the major mucoprotein component of normal human plasma has been described, and chemical and electrophoretic characterization of this substance was presented (4). In this communication, further studies of the physical properties of this mucoprotein will be reported.

Behavior in Ultracentrifuge

Sedimentation and diffusion studies were carried out with an electrophoretically homogeneous mucoprotein, having the chemical composition previously reported (4), isolated by fractionation with ammonium sulfate from pooled, normal, human plasma.

The sedimentation studies were made in the Spinco[1] electrically driven ultracentrifuge with the controls and procedures previously described (5). All of the runs were performed at 59,780 r.p.m., equivalent to centrifugal fields of approximately 240,000 $\times$ g and 300,000 $\times$ g at the meniscus and base.

In Fig. 1, Philpot-Svensson diagrams of the sedimenting mucoprotein are shown as observed in representative runs at different pH values. It is apparent that the substance behaves as a molecularly monodisperse system under these conditions. In fact, at no time was any evidence found for the presence of other sedimenting boundaries.

Individual determinations of the sedimentation constants are shown in Fig. 2. The values are given in Svedberg units (S = 1 $\times$ 10^{-13}),

[1] Specialized Instrument Corporation, Belmont, California.

and these are corrected for the density and viscosity of the medium (6). The temperature of each run was taken as the average for the time when photographs were made, and the results were then corrected to 20°. It is quite clear that the protein concentration has a significant effect on $s_{20,w}$, indicating a somewhat asymmetrical molecule. However, this effect
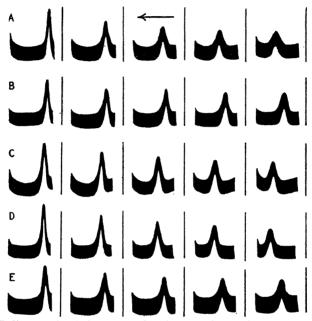
Fig. 1. Sedimentation behavior in the ultracentrifuge of a mucoprotein from human plasma. The arrow shows the direction of radial migration. A, results at pH 4.1 (0.1 M acetate + 0.1 M sodium chloride); first picture 36 minutes after attaining full speed, subsequent exposures at 32 minute intervals; protein concentration 0.92 per cent. B, the solvent was 0.15 M sodium chloride (pH 5.4); protein concentration 1.0 per cent; exposures 30 minutes after reaching full speed and at 16 minute intervals. C, the solvent was 0.1 M veronal + 0.4 M sodium chloride at pH 8.4; protein concentration 1.43 per cent; exposures 58 minutes after reaching full speed and at 32 minute intervals. D, results in 0.1 M glycine buffer at pH 9.5; protein concentration 1.14 per cent; exposures 38 minutes after reaching full speed and at 32 minute intervals. E, in 0.1 M glycine buffer at pH 10.5; protein concentration 0.50 per cent; exposures 32 minutes after reaching full speed and at 16 minute intervals.

seems to depend on the nature of the solvent. There appears to be some influence of ionic strength at the higher protein concentrations. A single determination (not plotted in Fig. 2) in 0.1 M veronal at pH 8.4 gave a value of $s_{20,w} = 2.68$ S at a protein concentration of 1.5 per cent. At an ionic strength of 0.5, $s_{20,w} = 2.94$ S. This effect of ionic strength was not observed at protein concentrations below 1 per cent.

Extrapolated values for zero protein concentration gave $s_{20,w} = 3.00$ S at pH 4.1, 3.16 S at pH 5.6, 3.06 S at pH 8.4, and 3.20 S at pH 9.5. The two determinations at pH 10.5 gave a somewhat higher value, but no significance can be attributed to these measurements at the present time.
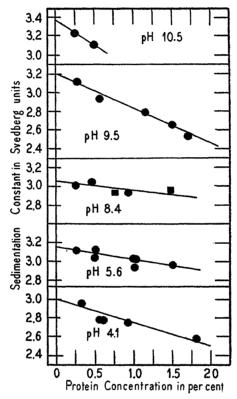
FIG. 2. Variation of the sedimentation constant ($s_{20,w}$) in Svedberg units as a function of the protein concentration as determined at different pH values. The measurements at pH 4.1 were made in an acetate buffer which contained 0.1 M acetate and 0.1 M sodium chloride.

Runs were made in 0.1 M veronal buffer at pH 8.4 (●), and in the same buffer supplemented with 0.4 M sodium chloride (■). The solvents at pH 9.5 and 10.5 were 0.1 M glycine buffers.

The average of the extrapolated values at the four lowest pH values is 3.11 S.

Diffusion Studies

The electrophoresis cell was used for the measurement of the diffusion constants from photographs taken by the Schlieren scanning method by

the method described by Longsworth (7). The results were computed by the formula $D = A^2/(4\pi tH^2)$ where A is the area under the curve, H the maximum height, t the time in seconds, and D the diffusion constant in sq. cm. per second.

Two runs were performed in veronal buffer of 0.1 ionic strength at pH 8.5. The measurements were made at 1.5° and corrected for the difference in viscosity and temperature in the usual manner (7). Each run was performed in duplicate, both halves of the cell being used separately. Six or seven photographs were taken at intervals of 8 to 70 hours and the values averaged. The data are given in Table I.

The average diffusion constant found for the mucoprotein is 5.27 ± 0.06 × 10⁻⁷ sq. cm. per second. It is apparent that, at the two values studied, the protein concentration has no detectable effect on the diffusion constant.

TABLE I

Diffusion Constants of Plasma Mucoprotein

Protein concentration	$D_{1.5°,v} \times 10^7$	$D_{20,w} \times 10^7$
per cent	*sq. cm. per sec.*	*sq. cm. per sec.*
0.98	2.895	5.40
0.98	2.82	5.27
0.39	2.79	5.21
0.39	2.79	5.21

Partial Specific Volume

These measurements were performed in 10 cc. pycnometers with a preparation of the mucoprotein that was about 95 per cent homogeneous electrophoretically at pH 4.0. Values of the partial specific volume ranging from 0.671 for a protein concentration of 1.275 per cent to 0.678 for a concentration of 0.380 per cent were obtained by the formula 110 given in Svedberg and Pedersen (6).

The average value of 0.675 for the apparent partial specific volume (V) of this mucoprotein is somewhat lower than that previously found for many proteins. However, it is very close to the value of 0.685 found by Fredericq and Deutsch (8) for ovomucoid, a protein which contains proportions of carbohydrate somewhat similar to those of the plasma mucoprotein (4).

Molecular Weight and Frictional Coefficient

Using the usual formula for the molecular weight (6), where $M = RTs/D(1 - V\rho)$, and substituting the values found for s, D, and V, we obtain 44,100.

The frictional coefficient, f/f_0, was calculated to be 1.78 from the sedimentation and diffusion constants (6). The maximal value of the axial ratio is about 15 for an elongated ellipsoid, or 20 for a flattened ellipsoid.

Viscosity Studies

Viscosity measurements were carried out on a preparation of the mucoprotein which was 90 per cent homogeneous by electrophoresis at pH 2.7, by means of an Ostwald viscosimeter mounted in a water bath at 25°. The results of this study are recorded in Table II. The values were extrapolated by means of the function $H = (\ln n/n_0)/C$, H being plotted *versus* C. The intrinsic viscosity, H_0, was taken as the extrapolated value of H at $C = 0$. Here n is the viscosity of the protein solution, n_0 the viscosity of the solvent (double distilled water), and C is the mucoprotein concentration in gm. per 100 cc. of solution. The value of the viscosity

TABLE II

Viscosity Studies of Plasma Mucoprotein

Concentration	Relative viscosity, n/n_0	$\ln n/n_0$	H
gm. per 100 cc.			
6.38	1.605	0.4731	0.0742
3.86	1.318	0.2761	0.0715
3.00	1.237	0.2127	0.0709
2.18	1.167	0.1544	0.0708
0	1.000	0	(0.069)

increment, ν, calculated from the intrinsic viscosity H_0 and the partial specific volume V_1 by the equation $\nu = 100\ H_0/V_1$, was found to be 10.2. This corresponds to an axial ratio of about 8 for an elongated ellipsoid or approximately 13 for a flattened ellipsoid. The agreement between the axial ratios obtained from viscosity studies and those obtained from diffusion and sedimentation is not particularly good. If the calculations are made on the basis of a hydrated protein containing 0.2 gm. of water per gm. of protein, the discrepancy becomes somewhat less significant, especially for a flattened ellipsoid, the values being 10 from viscosity measurements and 15 from diffusion measurements ($f/f_0 = 1.63$).

DISCUSSION

It has previously been pointed out (4) that this mucoprotein does not appear to bear any easily recognized relationship to the well known protein constituents of human plasma. The high content of carbohydrate, the low isoelectric point (pH 1.8), the amino acid composition, and the

presence of labile sulfate have all served to distinguish this protein from other plasma proteins. The present studies support the earlier conclusions.[2] The sedimentation and diffusion constants of the mucoprotein are quite different from those found for other constituents of human plasma (9). The molecular weight of 44,100 establishes this protein as one of the smallest proteins present in human plasma. Of considerable interest is the low partial specific volume (0.675) of the mucoprotein. It is suggested that this low value arises from the high carbohydrate content, since similar results have been found with ovomucoid (8).

It is of some interest to compare the molecular weight of the mucoprotein as determined by physical measurements with that computed from the amino acid analysis of Weimer, Mehl, and Winzler (4). Six of the

TABLE III

Molecular Weight of Mucoprotein from Amino Acid Content

Amino acid	Content	Minimum mol. wt.	Residues	Mol. wt.
	per cent			
Cystine	0.59	40,700	1	40,700*
Glycine	0.80	9,380	5	46,900
Histidine	1.28	12,100	4	48,400
Methionine	0.64	23,300	2	46,600
Tryptophan	1.22	16,700	3	50,100
Tyrosine	1.95	9,290	5	46,450
Average				46,500

* This low value suggests that some cystine is destroyed during hydrolysis.

amino acids are present in small enough amounts to permit such calculations. The data in Table III are calculated from the amino acid figures given for the anhydrous substance; these lead to an average molecular weight of 46,500 which is fairly satisfactory.

As already pointed out (4), methionine and cystine do not account for all of the sulfur, 1.02 per cent, which is present in the mucoprotein. Cal-

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[2] It was hoped that further characterization of the mucoprotein could be achieved by immunochemical methods. Preliminary experiments have been made by Dr. B. V. Jager of the University of Utah. Attempts were made to stimulate antibody production by repeated injection into rabbits and chickens of alum-precipitated preparations of the mucoprotein. This was tried at 50 to 100 mg. of protein per kilo of body weight. Thus far, none of these efforts has been successful. However, these negative experiments do serve to distinguish the poorly antigenic mucoprotein from other human plasma proteins which are highly antigenic. Antisera to human γ-globulin and to human albumin gave no precipitates with the mucoprotein.

culation gives 14 or 15 sulfur atoms, while only 4 can be present in the cysteine and methionine residues. It is apparent that 10 or 11 sulfur atoms are present in another form, probably as labile sulfate esters.

SUMMARY

Examination of a purified mucoprotein from human plasma showed that it behaved as a homogeneous substance in the ultracentrifuge over the range of pH 4.1 to 10.5. The average sedimentation constant ($s_{20,w}$) is 3.11 S, as found by extrapolation to zero protein concentration. The average diffusion constant is 5.27×10^{-7} sq. cm. per second. The apparent partial specific volume is 0.675. The intrinsic viscosity is 0.069. The computed molecular weight is 44,100, and the frictional coefficient is 1.78.

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ON DEETHYLATION OF ETHIONINE IN THE RAT*

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Research, Philadelphia)

We reported recently that ethionine (the ethyl analogue of methionine) inhibits the growth of rats and that either choline or methionine alleviates the inhibition (1). In discussing these observations we advanced the hypothesis that ethionine administration increases the demand either for choline *per se* or for the "labile methyl" group which is utilized by the animal for methylations to yield such vital metabolites as creatine, choline, epinephrine, etc. (1). The apparent specificity of the "labile methyl" group donors in the alleviation of the inhibition of growth by ethionine suggested the interesting possibility of involvement of the ethyl group of ethionine by replacement of the methyl group in metabolic methylations. Such a possibility implies deethylation of ethionine *in vivo* and the participation of the ethyl group directly or indirectly in what is known as transmethylation reactions. Should such replacement of the methyl group by the ethyl of ethionine occur, it is conceivable that the resulting ethyl analogues of essential metabolites can no longer function in the manner their methyl counterparts do under normal circumstances. The inevitable result is interference with normal metabolic processes which may at least in part account for the cessation of growth.

In the present work we tested the possibility of deethylation of ethionine in the intact rat by employing ethionine which was labeled with C^{14} in the methylene carbon of the ethyl group and S^{35}-ethionine. From the tissue of rats which were given C^{14}-ethionine we isolated choline and creatinine. Both compounds contained radioactive carbon. From the urine of rats which received S^{35}-ethionine and bromobenzene we isolated radioactive p-bromophenylmercapturic acid. The radioactivity of choline was confined to the trimethylamine portion of the molecule which was obtained on degradation of the isolated choline.

EXPERIMENTAL

C^{14}-Ethionine was synthesized by ethylation of the sodium salt of homocysteine in liquid ammonia with ethyl iodide, which was labeled with

C^{14} in the methylene carbon. The specific activity of C^{14}-ethionine was 3.72×10^5 c.p.m. per mg. S^{35}-Ethionine was prepared by ethylation of the sodium salt of S^{35}-homocysteine in liquid ammonia with ordinary ethyl iodide. S^{35}-Homocystine was synthesized from S^{35}-thiourea by an adaptation of the procedure of Snyder et al. (2). S^{35}-Thiourea was obtained from the Abbott Laboratories. The specific activity of S^{35}-ethionine was 1.31×10^4 c.p.m. per mg. The radioactive ethionines were free of either the thiol or the disulfide group within the limits of accuracy of the tests applied (sodium cyanide-nitroprusside and phosphotungstic acid reactions). Two very faint spots, in addition to the spot given by ethionine, were, however, obtained on a chromatogram. Both of the extra spots were weakly radioactive in the case of S^{35}-ethionine, and only one extra spot was weakly radioactive in the case of C^{14}-ethionine. One of the extra spots, which was weakly radioactive in both cases, corresponded to that given by ethionine sulfoxide which was probably formed during the development of the chromatogram. The other spot was apparently that of homocystine. After the result with chromatograms was obtained, both radioactive ethionines were again recrystallized three times from dilute ethanol, and the resulting products were used in animal experiments.

One adult female rat and one pregnant rat of Wistar strain were used with C^{14}-ethionine. 10 mg. of C^{14}-ethionine, dissolved in water, were injected intraperitoneally into each rat. A complete 25 per cent casein diet and water were allowed ad libitum. 6 days later the food was withdrawn from the cage, and the rat was fasted 2 days and then sacrificed. 4 days after the administration of C^{14}-ethionine into the pregnant rat, 4 pups were born. The pups were removed from the cage within 1 hour after delivery, washed with soap and water, and sacrificed. The mother was fasted for 2 additional days, then sacrificed. The skin was removed from both adult rats, and each rat (after washing the intestines with water) was ground up separately in a meat chopper. The pups were ground up after being washed. Choline was isolated from each rat and the pups as the reineckate, then converted to chloroplatinate (3), and creatine was isolated as creatinine-potassium picrate (4). After the specific activities of isolated products were determined, the compounds were subjected to elementary analysis. The remaining portion of choline chloroplatinate was degraded to trimethylammonium chloride (3) and the specific activity of the salt was determined.

5 mg. of S^{35}-ethionine and 100 mg. of bromobenzene were injected intraperitoneally into two other rats which were subsisting on a 25 per cent casein diet and water ad libitum. The urine of each rat was collected separately for the next 3 days, made up to a convenient volume, and an aliquot was used for the determination of the total amount of the mercapturic

acid excreted (5). From the remainder of the urine p-bromophenylmercapturic acid was isolated, identified, and assayed for radioactivity. The compound was then deacetylated by refluxing for 3 hours in 6 N HCl, and the resulting p-bromophenyl-L-cysteine was isolated and assayed for radioactivity.

In order to rule out the possibility of contamination of our isolated materials by unchanged C^{14}-ethionine, 10 mg. of C^{14}-ethionine, dissolved

TABLE I

Radioactivity of Creatinine, Choline, and p-Bromophenylmercapturic Acid Isolated from Tissues and Urine of Rats on Administration of Either C^{14}-Ethionine or S^{35}-Ethionine and Bromobenzene

Rat No.	Compound administered	Compound isolated*	Radioactivity	
			c.p.m. per mM	*c.p.m. per mM "methyl"*
14	C^{14}-Ethionine	Choline reineckate	7,832	2611
		" chloroplatinate	15,498	2583
		Trimethylammonium chloride	8,903	2968
		Creatinine-K picrate	2,367	2367
Pups of Rat 14	" to mother	Choline reineckate	6,552	2184
		" chloroplatinate	14,346	2391
		Trimethylammonium chloride	7,200	2400
		Creatinine-K picrate	2,114	2114
17	C^{14}-Ethionine	Choline reineckate	9,000	3000
		" chloroplatinate	17,430	2905
		Trimethylammonium chloride	8,900	2967
		Creatinine-K picrate	2,450	2450
15	S^{35}-Ethionine	p-Br-phenylmercapturic acid	3,510	
		p-Br-phenyl-L-cysteine	3,448	
16	"	p-Br-phenylmercapturic acid	3,666	
		p-Br-phenyl-L-cysteine	3,710	

* The analytical values of the isolated compounds were as follows:
Choline chloroplatinate.   Calculated, Pt 31.7; found, Pt 31.0 ± 0.1
Creatinine-K picrate.   "   N 20.7; "   N 20.2 ± 0.2
p-Br-phenyl-L-cysteine (m.p. 182–183°)   "   " 5.02; "   " 4.98

in water, were added to ground up tissues of a stock rat, and creatinine-potassium picrate and choline reineckate were isolated as described. Both compounds were free of radioactivity.

The radioassays were made in a Geiger-Müller counter with a mica window of 1.8 mg. per sq. cm. on weighed samples of the products which were spread over an area of 5 sq. cm. Corrections for background count and decay of S^{35} were made. We are aware that a higher precision of radioassay could have been secured by conversion of the C^{14} compounds to

CO_2 and the S^{35}-containing compounds to sulfates, and determination of the radioactivity on the carbonates and the sulfates respectively. The purpose of the present work, however, was merely to establish significant radioactivity of the isolated products, without consideration of the quantitative aspect of the incorporation of the radioactive atoms into the isolated material.

Results

The data are summarized in Table I. It is clear that the methylene carbon of the ethyl group of ethionine found its way into creatinine and choline of rat tissues. The activity of this carbon was in the trimethylamine moiety of the choline molecule. The data also show that ethionine sulfur is available to the rat for cysteine synthesis. Even if we assume that some radioactive homocysteine was present in the administered S^{35}-ethionine as an impurity, which we failed to detect, the magnitude of the activity of the mercapturic acid was such as only grossly contaminated ethionine could produce. The diet contained 25 per cent of casein, and the extent of dilution of the cysteine within the rat must have been very large. As could, perhaps, be anticipated, the tissue of new-born rats also yielded radioactive choline and creatinine.

DISCUSSION

The data at hand, although highly suggestive, do not prove beyond doubt that the ethyl group of ethionine was actually present in the choline and creatinine molecules. Attempts to prove or disprove the presence of the ethyl group in these metabolites will be made. At this time we can only summarize the existing evidence in favor of the possibility that the ethyl group *per se* was incorporated in the molecules of creatinine and choline, and probably in other metabolites which are involved in transmethylation reactions.

Cysteine formation from the sulfur of ethionine suggests beyond reasonable doubt the formation of homocysteine from ethionine in the rat, if one accepts the mechanism that cysteine formation in the rat involves the condensation of homocysteine with serine to yield cystathionine which, on cleavage, yields cysteine (6). This method of cysteine formation calls for deethylation of ethionine. The exact mechanism of removal of the methyl group from methionine is not clear, and we must admit it is equally obscure in the case of ethionine. The transfer of either the methyl of methionine or of the ethyl of ethionine may have been direct in the presence of the acceptors, or it may have been preceded by removal of these groups as corresponding alcohols. Since the methyl group of sarcosine underwent oxidation to formaldehyde in rat liver preparations (7), the possibility of

cleavage of the methyl group of methionine as methanol and, by analogy, of the ethyl group of ethionine as ethanol is an interesting possibility worth testing experimentally.[1]   However, it appears improbable that in our experiments the ethyl group of ethionine was transformed *in vivo* to the methyl group, which then followed the path which was charted for it by the richly documented data of du Vigneaud and associates.   If such were the case, it is difficult to explain the apparent unavailability of ethionine to the rat for growth purposes in lieu of methionine (8), its toxicity (9), and the alleviation of the growth inhibition induced by ethionine by "labile methyl" group donors (1).   One might conceivably wonder at this point why ethionine is not available to the rat for growth purposes in lieu of methionine if, as we propose, ethionine yields homocysteine *in vivo*.   In the presence of dietary choline the resulting homocysteine should yield methionine.   It should be borne in mind that the extent of such homocysteine formation from ethionine may be and probably is too small to yield large enough amounts of methionine to replace the entire requirements of methionine for growth.[2]   There is no doubt that a considerable portion of administered ethionine is excreted either unchanged or in some modified form (10).[3]

Further evidence for the interference by the ethyl group in physiological processes is furnished by the data of Keston and Wortis (11) who reported that the triethyl analogue of choline interferes with the acetylcholine formation in the mouse.   This effect of triethylcholine could be abolished by choline.   We also found that triethylcholine inhibits the growth of rats and the inhibition could be alleviated either by choline or, less effectively, by methionine (12).

These considerations argue strongly in favor of the possibility that the radioactive creatinine and choline, which we isolated from the tissues of rats on administration of radioethionine, contained the intact ethyl group

[1] In such a case the ethyl group of ethionine should be available *in vivo* as acetic acid on the assumption that oxidation of ethanol to acetic acid occurs via acetaldehyde.   Such a sequence of events does not preclude the possibility of condensation reactions *in vivo* in which the carbons of the ethyl group may appear in metabolites.   One such reaction, for example, is the acetylation reaction in the formation of the mercapturic acids.

[2] On a diet consisting of a mixture of amino acids containing 0.2 to 0.25 per cent of methionine, 0.5 per cent of choline, 0.5 per cent of ethionine, and supplemented with 10 γ of vitamin B_{12} per 100 gm. of food, the rats continue to grow at a rate which is comparable to or better than that obtained on the same diet free of ethionine (Stekol and Weiss, unpublished data).

[3] The excretion of radioactivity in the urine of rats, to which was administered either C^{14}-ethionine or S^{35}-ethionine, extends over a period of several weeks.   During this extended period only a fraction of the radioactivity of urine of S^{35}-treated rats is in the inorganic sulfates (Stekol and Weiss, unpublished data).

of ethionine. The formation of these ethyl analogues *in vivo*, and probably others, was at least in part responsible for the interference with the normal metabolic processes which resulted in cessation of growth. The simultaneous administration of either choline or methionine, and probably other "labile methyl" group donors, with ethionine reduces or competes with the synthesis of these ethyl analogues, thereby alleviating the inhibition effect. Such a hypothesis does not necessarily imply that the ethyl analogues of creatine or creatinine or of other methyl group acceptors will as a rule produce the same effect when they are administered directly. Such seems to be the case with triethylcholine. The situation prevailing when such an analogue is synthesized *in vivo* at the expense of the methyl counterpart is somewhat different from that when the analogue is administered directly if we take into consideration the site of its formation, the form in which it exists *in vivo*, and the relationship to other metabolites from which it is formed. Nevertheless, an evaluation of ethyl analogues of such substances as epinephrine, *N*-methylnicotinamide, sarcosine, etc., appears to be of some interest.

Recently Tarver *et al.* (13) reported that ethionine reduced the incorporation of either S^{35}-methionine or C^{14}-glycine into rat liver proteins, which was interpreted as an indication of inhibition of protein synthesis by ethionine. The authors stated that such an interpretation of their data is valid only on the assumption that ethionine does not undergo deethylation in the rat. In view of the results reported here and because of the fact that glycine carbons and the carbon of the methyl group of methionine are precursors of the carbon chain of cysteine in the rat (14), the data of Tarver *et al.* (13) do not necessarily argue for or against the rôle of ethionine in the synthesis of protein in the rat as measured by the uptake of the isotopic tracers.

SUMMARY

1. Ethionine, which was labeled with C^{14} in the methylene carbon of the ethyl group, was administered to rats and radioactive creatinine and choline were isolated from the tissues. The radioactivity of choline was confined to the trimethylamine moiety of the molecule.

2. S^{35}-Ethionine was administered to rats together with bromobenzene, and radioactive *p*-bromophenylmercapturic acid was isolated from the urine.

3. The data suggest that ethionine sulfur is available to the rat for cysteine synthesis, implying deethylation of ethionine *in vivo*, and that the ethyl group of ethionine is available to the rat for the synthesis of ethyl analogues of substances which are known to participate in the so called transmethylation reactions. It is suggested that the observed inhibition

of growth by ethionine is probably due to the synthesis of these ethyl analogues *in vivo*.

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THE INHIBITION OF GROWTH OF RATS BY TRIETHYLCHOLINE*

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In the preceding paper evidence was presented for the incorporation of the ethyl group carbon of ethionine into choline and creatinine of rat tissue (1). These observations suggested the formation of ethyl analogues of essential metabolites *in vivo* by replacement of the methyl group by the ethyl of ethionine in transmethylation reactions. It was further suggested that the formation of such ethyl analogues *in vivo* may at least in part account for the inhibition of growth of rats on administration of ethionine (1).

It appeared of interest to test the effectiveness of the ethyl analogue of choline, triethylcholine, on the growth of rats and to attempt to alleviate the inhibition by either choline or methionine. It has been reported that triethylcholine is toxic to mice and that the toxicity could be alleviated by choline. This effect of triethylcholine on mice was attributed to its interference with acetylcholine formation (2).

EXPERIMENTAL

The growth experiments were carried out on two diets. Diet C-25 had the following per cent composition: Labco vitamin-free casein 25, sucrose 15, corn-starch 31, Crisco 20, U. S. P. XII salt mixture 4, and cod liver oil 5. Diet C-8 contained 8 per cent of casein instead of 25, with appropriate adjustment of the starch content. 1 kilo of each diet was supplemented with thiamine hydrochloride 10 mg., riboflavin 20 mg., pyridoxine hydrochloride 10 mg., calcium pantothenate 50 mg., biotin 1 mg., nicotinamide 10 mg., p-aminobenzoic acid 1000 mg., inositol 1000 mg., folacin 10 mg., methylnapthoquinone 10 mg., and α-tocopherol acetate 30 mg. Rats of Wistar strain of about 35 days of age, born and raised in the laboratory, were kept in individual cages with raised screened floors. Food and water were allowed *ad libitum*. Triethylcholine was generously supplied by Merck and Company and racemic methionine by United States Industrial Chemicals, Inc., through Dr. H. Prebluda. We wish to express our thanks for the support given us.

Results

The data obtained on eleven groups of male and·female rats, six animals per group, are summarized in Table I. It is evident that 0.5 per cent of triethylcholine in either diet inhibited the growth of rats and that the addition of 0.5 per cent of choline chloride to the supplemented diet alleviated the inhibition. An equal weight of methionine added to either diet which contained 0.5 per cent of triethylcholine also alleviated the inhibition of growth but to a considerably smaller degree.[1] The livers of all rats, with the exception of the controls which were on Diet C-8, were not fatty. The

TABLE I

Inhibition of Growth of Rats by Triethylcholine and Its Alleviation by Either Choline or Methionine

Group No.*	Diet	Supplements			Gain in weight	Days on diet
					gm.	
1 ♂	C-8	0.5% triethylcholine			-2.0 ± 0.8	30
2 ♂	C-8	0.5% "	and 0.5% choline		21.5 ± 1.0	30
3 ♂	C-8	0.5% "	" 0.5% methionine		11.0 ± 1.2	30
4 ♀	C-8	0.5% "			-11.0 ± 2.0	30
5 ♀	C-8	0.5% "	and 0.5% choline		20.0 ± 2.0	30
6 ♀	C-8	0.5% "	" 0.5% methionine		8.0 ± 1.4	30
7 ♂	C-8	None			27.0 ± 2.0	30
8 ♂	C-25	0.5% triethylcholine			-3.0 ± 1.5	20
	C-25	0.5% "	and 0.5% choline		50.0 ± 2.7	10
9 ♂	C-25	0.5% "	" 0.5% "		85.0 ± 3.0	23
10 ♂	C-25	0.5% "	" 0.5% methionine		29.0 ± 2.0	23
11 ♂	C-25	None			92.0 ± 3.5	23

* Each group consisted of six rats. The gain in weight per rat during the period is the average. The data on food consumption were not included in the table, as they appeared of little value in interpreting the results. Choline was administered as choline chloride.

availability of triethylcholine for lipotropism has been reported previously (3).

Evidence has been reported for the replacement of choline by triethylcholine as a component of the liver lecithins (4). Interference of triethylcholine with the synthesis of acetylcholine in the mouse (2) has already been mentioned. It is possible, therefore, that the inhibition of growth by

[1] It is realized that this amount of methionine contains fewer "labile methyl" groups than the same weight of choline, even if only one methyl group of choline is available. However, it would be premature to conclude that this fact would account for the results obtained.

triethylcholine may have been a result of replacement of choline by triethylcholine in metabolic functions in which choline plays a rôle. Of these functions, that of transmethylation seems particularly of interest in connection with our experiences with ethionine (1). Pending proof we are inclined to assume that the ethyl group of triethylcholine, like that of ethionine, might be available *in vivo* in lieu of the methyl group in transmethylation reactions. The formation of the ethyl analogues, as a result of such replacement of the methyl group by the ethyl of triethylcholine, may be another contributing factor in the phenomenon of the inhibition of growth.

SUMMARY

The ethyl analogue of choline, triethylcholine, inhibited the growth of rats. The inhibition was alleviated either by choline or, less effectively, by methionine. The possibility of the involvement of the ethyl group of triethylcholine in transmethylation reactions is pointed out.

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STEROIDS DERIVED FROM BILE ACIDS

IX. DIPHENYLCARBINOL AND DIPHENYLETHYLENE DERIVATIVES*

By VERNON R. MATTOX and EDWARD C. KENDALL

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Introduction of a ketone group at C-11 in methyl 3,9-epoxy-11,12-dibromocholanate and the cleavage of the 3,9-epoxide to reestablish the 3-hydroxyl group have been described (1). For the further steps in the conversion of desoxycholic acid to the cortical hormones it is necessary to degrade the side chain to a ketone group at C-20 and to add a hydroxyl group at C-21. It has been found that both of these procedures can be carried out in good yield subsequent to formation of a 24,24-diphenyl-Δ^{23}-cholene which is made through the addition of phenyl magnesium bromide to the C-24 methyl ester of the bile acid derivative, followed by dehydration of the resulting carbinol.

A question of some importance arose concerning which intermediate compound should be used for formation of the diphenyl-Δ^{23}-cholene. This could be formed from either methyl 3(α)-hydroxy-11-keto-12(α)-bromocholanate (1) or from methyl 3,9-epoxy-11-keto-12(α)-bromocholanate (1). With the 3(α)-hydroxy compound it was found that addition of the Grignard reagent resulted in formation of a product gelatinous in nature and only partially soluble in the benzene-ether solution. The 3,9-epoxy compound afforded a Grignard reaction product which was completely soluble and the yield of the carbinol was high.

For the preparation of the 24,24-diphenyl derivative of the 3,9-epoxy compound the only departure in application of the usual method (2) was that the addition of methyl 3,9-epoxy-11-keto-12(α)-bromocholanate to phenyl magnesium bromide was made at 0° to −30° instead of at room temperature or above. The atom of bromine at C-12 adjacent to the keto group was removed by the Grignard reagent (3) and did not require a separate step. The carbinol was converted into 3,9-epoxy-11-keto-24,24-diphenyl-Δ^{23}-cholene almost quantitatively by dehydration with boiling acetic acid.

There remained the question of whether the diphenyl-Δ^{23}-cholene could be treated successfully with concentrated hydrogen bromide in order to open the 3,9-epoxy linkage. It has been pointed out previously (1) that the oxide ring in methyl 3,9-epoxy-11-ketocholanate is easily opened with

concentrated hydrogen bromide. As the side chain is shortened there is a progressive decrease in the reactivity of the 3,9-epoxide toward hydrogen bromide. In keeping with this observation it has been found that the oxide ring in 3,9-epoxy-11-keto-24,24-diphenyl-Δ^{23}-cholene is easily opened with concentrated hydrogen bromide in chloroform at 0°. After acetylation of the 3(α)-hydroxyl group, the yield of 3(α)-acetoxy-11-keto-12(α)-bromo-24,24-diphenyl-Δ^{23}-cholene from the 3,9-epoxy compound was about 90 per cent of the theoretical amount.

We wish to acknowledge the able technical assistance of Mr. Curtis Dunlap.

<div align="center">EXPERIMENTAL</div>

All melting points were taken on a Fisher-Johns apparatus and recorded as read. All rotations were taken at a concentration of approximately 1 gm. per 100 ml. of solution. The compounds described in this paper were analyzed in the laboratory of Merck and Company, Inc., Rahway, New Jersey.

3,9-Epoxy-11-ketonorcholanyldiphenylcarbinol—A solution (315 ml.) of 2.28 N phenyl magnesium bromide (6 equivalents) in ethyl ether was cooled in an ice-salt bath and a solution of 57.62 gm. of methyl 3,9-epoxy-11-keto-12(α)-bromocholanate in 575 ml. of dry benzene was added with stirring. The temperature was maintained close to 0°. After 16 hours at 0° the solution was poured on chipped ice and 115 gm. of ammonium chloride. After the organic phase had been washed with dilute hydrochloric acid, dilute sodium carbonate solution, and water it was concentrated under reduced pressure and 250 ml. of methanol were added. The carbinol which separated (50.94 gm.) melted at 164–166°. 10 ml. of 5 N aqueous sodium hydroxide were added to the filtrate and a rapid flow of steam was then passed through the solution to remove bromobenzene and diphenyl. The supernatant liquid was decanted from the insoluble material which was dissolved in a few ml. of benzene, methanol was added, and the solution was concentrated on the steam bath. A second crop (5.37 gm.) melted at 162–164°. A sample which had been recrystallized several times from acetone-water melted at 167–168°. $[\alpha]_D = +67° \pm 2°$ (chloroform).

Analysis—$C_{36}H_{46}O_3$. Calculated, C 82.08, H 8.80; found, C 81.80, H 8.50

3,9-Epoxy-11-keto-24,24-diphenyl-Δ^{23}-cholene—A solution of 3,9-epoxy-11-ketonorcholanyldiphenylcarbinol (65.75 gm.) in 660 ml. of glacial acetic acid was refluxed for 2 hours and cooled; crystals formed. Yield 57.75 gm., m.p. 153–154°. A second crop of 3.96 gm. separated after concentration of the mother liquor, m.p. 149–152°. Crystallization from chloro-

form-methanol gave material which melted at 153–154°. $[\alpha]_D = +83°$ ± 2° (chloroform).

Analysis—$C_{36}H_{44}O_2$. Calculated, C 84.99, H 8.72; found, C 84.70, H 8.72

Within an interval of 40 minutes, a solution of 240 gm. of methyl 3,9-epoxy-11-keto-12(α)-bromocholanate in 750 ml. of dry benzene was added to 1450 ml. of an ethereal solution of 2.04 N phenyl magnesium bromide. The flask containing the Grignard reagent was cooled in a dry ice-acetone bath to −25° to −30° during addition of the ester. After 90 minutes, during which the temperature rose to 0°, the solution was poured on cracked ice to which had been added 390 ml. of concentrated hydrochloric acid. The organic phase was separated, the ether contained in the solution of the Grignard reagent was evaporated, 400 ml. of benzene were added, and after the benzene solution had been vigorously stirred for 30 minutes with 100 gm. of sodium hydroxide flakes it was filtered through a pad of infusorial earth and evaporated to dryness. 1000 ml. of acetic acid were added and the solution was refluxed for 3 hours. When the solution was cooled, 210 gm. of material, m.p. 154.5–155°, separated. A second crop of 13 gm. was obtained when the mother liquor was concentrated to a small volume, m.p. 149–151°. The acetic acid was removed and a rapid flow of steam was passed through the flask to remove diphenyl and bromobenzene. The residue was extracted with benzene and the solution was evaporated to dryness. A third crop of crystals (6 gm., m.p. 149–151°) was obtained from acetic acid.

3(α)-Acetoxy-11-keto-12(α)-bromo-24,24-diphenyl-Δ^{23}-cholene—50 gm. of 3,9-epoxy-11-keto-24,24-diphenyl-Δ^{23}-cholene and 60 ml. of dry chloroform were placed in a pressure bottle. The solution was cooled in a dry ice bath and 125 gm. of dry hydrogen bromide were added. The flask was sealed and placed in an ice bath. After 48 hours at 0° the flask was cooled in a dry ice bath, opened, and the contents poured on chipped ice. Seven such batches were combined, chloroform was added, and the solution was washed well with water, dried with sodium sulfate, and concentrated to dryness under reduced pressure. The last of the chloroform was displaced with acetic anhydride and the solution was refluxed at a volume of 500 ml. for 3 hours. The acetate separated upon cooling. Yield 393 gm. (90 per cent), m.p. 178–179°. $[\alpha]_D = +18° \pm 2°$ (chloroform).

Analysis—$C_{38}H_{47}O_3Br$. Calculated. C 72.25, H 7.50, Br 12.65
Found. " 72.20, " 7.48, " 12.83

SUMMARY

The treatment of methyl 3,9-epoxy-11-keto-12(α)-bromocholanate at low temperatures, −30° to 0°, with phenyl magnesium bromide removes

the atom of bromine at C-12 and forms the 24,24-diphenylcarbinol in excellent yield. The diphenylcarbinol is dehydrated in boiling acetic acid to give 3,9-epoxy-11-keto-24,24-diphenyl-Δ^{23}-cholene almost quantitatively.

The 3,9-epoxy linkage of the diphenyl-Δ^{23}-cholene is opened with hydrogen bromide at 0° and the resulting 3(α)-hydroxy-11-keto-12(α)-bromo derivative is acetylated in acetic anhydride to give 3(α)-acetoxy-11-keto-12(α)-bromo-24,24-diphenyl-Δ^{23}-cholene.

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STEROIDS DERIVED FROM BILE ACIDS

X. PREPARATION OF BROMO DERIVATIVES OF SOME 3-KETOSTEROIDS*

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For the introduction of a double bond at 4-5 in 3-ketosteroids it became necessary to prepare the 4-bromo derivatives of these compounds. Methyl 3,12-diketocholanate was first used as a model substance, but this compound was found unsuitable because of its failure to form easily crystallizable products after bromination. Methyl 3,11-diketo-12(α)-bromocholanate (1, 2) proved to be starting material which was satisfactory.

In acetic acid 1 mole of bromine was consumed and the 4,12(α)-dibromo compound was formed as the principal product. This was accompanied by small amounts of a 2,4,12(α)-tribromo compound if the solution was allowed to stand a few minutes in the presence of hydrogen bromide after bromination was complete. However, it was found that, during an interval of 3 days in acetic acid which contained hydrogen bromide, disproportionation of the 4,12(α)-dibromo compound occurred (3) and from the solution the 2,4,12(α)-tribromo compound, a 2,12(α)-dibromo derivative, and the 12(α)-monobromo compound were isolated. The 2,12(α)-dibromo compound was obtained as a 3,3-dimethyl acetal by crystallization from methanol. The position of the halogen atom in ring A of these compounds was determined through dehydrobromination (4) to give the corresponding unsaturated derivative.

In order to suppress formation of the tribromo compound either directly or through disproportionation, the maximal concentration of hydrogen bromide during the bromination was controlled as follows. The acetic acid solution of the steroid was made 0.025 N in respect to hydrogen bromide by addition of the halogen acid in acetic acid. Sodium acetate in an amount equal to the hydrogen bromide formed during the bromination was added to a solution of bromine in acetic acid. The solution of bromine was added slowly so that the sodium acetate never exceeded the concentration of hydrogen bromide formed. As soon as the bromine was consumed the acetic acid solution of the reaction mixture was poured into water to suppress the action of hydrogen bromide. Under these conditions very little of the tribromo compound was formed.

After 1 mole of bromine had been consumed, the model substance absorbed a second mole of bromine to form the 2,4,12(α)-tribromide without any noticeable decrease in the rate of bromination.

Good yields of 4-bromo derivatives were obtained from the bromination of 3,11,20-triketo-12(α)-bromo-21-acetoxypregnane[1] and 3,11,20-triketo-12(α),21-dibromopregnane.[1] However, 3,11,20-triketo-17(α)-hydroxy-21-acetoxypregnane[2] (5) gave a mixture from which two monobromo compounds were separated. Each of these compounds melted at about 200° with decomposition, and gave no depression upon admixture. The specific rotation of the main portion was +102° and that of the remainder was +51° in acetone. It was shown through dehydrobromination[3] that the compound with the higher specific rotation was the 4-bromo derivative and that the other was a 2-bromo compound. The configuration of the bromine at C-4 or at C-2 has not been determined.

In a study of the bromination of 3-ketosteroids, Butenandt and associates (6, 7) found that when rings A and B were in the trans configuration, the bromine entered the steroid at C-2 and that when rings A and B were cis, bromination occurred at C-4. In this investigation 3,11,20-triketo-17(α)-hydroxy-21-acetoxypregnane gave the 4-bromo derivative in about 70 per cent yield, and between 15 and 25 per cent of 2-bromo product was invariably formed, as calculated from the specific rotation of the isolated fractions.

EXPERIMENTAL

All melting points were taken on a Fisher-Johns apparatus and recorded as read. All rotations were taken at a concentration of approximately 1 gm. per 100 ml. of solution. The brominations were performed at room temperature. The compounds described in this paper were analyzed by Mr. Joseph F. Alicino, Metuchen, New Jersey, Mr. William Saschek, Kent Laboratory, Chicago, Illinois, and in the laboratory of Merck and Company, Inc., Rahway, New Jersey.

Methyl 3,11-Diketo-4,12(α)-dibromocholanate—Methyl 3,11-diketo-12(α)-bromocholanate (4.81 gm.) was dissolved in 40 ml. of glacial acetic acid by warming, the solution was cooled to room temperature, and 1.0 ml. of 1.0 N hydrogen bromide in acetic acid was added. 820 mg. of anhydrous sodium acetate were dissolved in 20.0 ml. of 1.0 N bromine in acetic acid. The bromine-sodium acetate solution was added slowly to the solution of steroid with vigorous shaking. Utilization of the bromine

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[1] The preparation of these compounds by degradation of 3(α)-acetoxy-11-keto-12(α)-bromo-24,24-diphenyl-Δ^{23}-cholene will be described in the near future.

[2] We are indebted to Merck and Company, Inc., for this compound.

[3] Mattox, V. R., and Kendall, E. C., unpublished data.

was rapid and complete. Water (400 ml.) was added without delay and the solution was extracted with benzene. The benzene solution was washed with water, a dilute solution of sodium carbonate, and again with water, and was concentrated under reduced pressure. Crystals formed upon the addition of methanol; yield 3.66 gm., m.p. 153–154°; 1.13 gm., m.p. 149–150°. After several crystallizations from methanol a sample was obtained which melted at 157.5–158.5°. $[\alpha]_D = +12° \pm 2°$ (chloroform).

Analysis—$C_{25}H_{36}O_4Br_2$. Calculated. C 53.58, H 6.48, Br 28.52
Found. " 53.56, " 6.69, " 28.5

When the bromination was carried out without the use of sodium acetate and the brominated product was allowed to stand for 5 minutes before addition of water, 3 per cent of 2,4,12(α)-tribromo compound was isolated.

Methyl 3,11-Diketo-12(α)-bromo-Δ^4-cholenate—A solution of 1.12 gm. of methyl 3,11-diketo-4,12(α)-dibromocholanate in 10 ml. of dry pyridine was heated under a reflux for 5 hours. After cooling, ether was added and the solution was washed with water, dilute hydrochloric acid, and water and was concentrated under reduced pressure to dryness. Crystals (504 mg.), m.p. 185–187°, were obtained from methanol. A sample purified from methanol melted at 190–191°. $[\alpha]_D = +29° \pm 2°$ (chloroform). $\lambda_{max.}$ (methanol) 238 mμ; ϵ 16,600.

Analysis—$C_{25}H_{35}O_4Br$. Calculated. C 62.62, H 7.36, Br 16.67
Found. " 62.44, " 7.57, " 16.77

Disproportionation of Methyl 3,11-Diketo-4,12(α)-dibromocholanate. Separation of Methyl 3,11-Diketo-2,4,12(α)-tribromocholanate—Methyl 3,11-diketo-4,12(α)-dibromocholanate (560 mg.) was dissolved in 10 ml. of acetic acid, 0.46 ml. of 2.27 N hydrogen bromide in acetic acid was added, and the solution was sealed under an atmosphere of carbon dioxide. After 68 hours at room temperature, crystals (96 mg., m.p. 205–207°) were filtered off. The filtrate was distributed between chloroform and water, the organic phase was evaporated under reduced pressure, and crystals were obtained from acetic acid-ligroin. These crystals were combined with the 96 mg. and recrystallized from chloroform-methanol to give 106 mg. of product, m.p. 210–212°, that did not depress the melting point of methyl 3,11-diketo-2,4,12(α)-tribromocholanate. $[\alpha]_D = -33° \pm 2°$ (chloroform).

Separation of Methyl 11-Keto-2,12(α)-dibromo-3,3-dimethoxycholanate—Attempts to isolate other compounds from the acetic acid-ligroin mother liquor of the preceding paragraph were unsuccessful until methanol was tried. Crystals separated after the residue had remained 1 week in 5 ml. of methanol and were filtered off after 3 weeks yield, 172 mg.,

m.p. 122–126°. Several crystallizations from methanol raised the melting point to 125–127°. $[\alpha]_D = -62° \pm 2°$ (chloroform).

Analysis—$C_{27}H_{42}O_5Br_2$. Calculated.   C 53.47, H 6.98, CH_3O 15.35
Found.   " 53.48, " 6.96,   "   14.81

Evidence for the position of the atom of bromine in ring A is given in paper XI (4).

Separation of Methyl 3,11-Diketo-12(α)-bromocholanate—In another experiment in which 4.38 gm of methyl 3,11-diketo-4,12(α)-dibromocholanate were treated as described in the previous two paragraphs, an oil remained after separation of the methyl 11-keto-2,12(α)-dibromo-3,3-dimethoxycholanate. The oil was dissolved in 40 ml. of 90 per cent acetic acid to hydrolyze the dimethyl acetal at C-3. After 24 hours water was added, the solution was concentrated under reduced pressure, the residue was extracted with chloroform, the organic phase was evaporated, and crystals (256 mg., m.p. 140–142°) were obtained from ether-ligroin. After recrystallization from ether the product melted at 157–158° and did not depress the melting point of methyl 3,11-diketo-12(α)-bromocholanate. $[\alpha]_D = +21° \pm 2°$ (acetone) (1).

Methyl 3,11-Diketo-2,4,12(α)-tribromocholanate—Methyl 3,11-diketo-12(α)-bromocholanate (1.92 gm.) was dissolved in 25 ml. of acetic acid and 15.4 ml. of 1.04 N bromine in acetic acid were added slowly with shaking. There was no noticeable change in the rate of consumption of bromine after 1 mole had been added. The solution became colorless within 1 minute after all of the bromine had been added, but was allowed to stand at room temperature for a half hour. Water was added, the solution was extracted with benzene, and the organic phase was washed with water and concentrated under reduced pressure until crystals formed. Yield 1.65 gm., m.p. 209–211°; 0.25 gm., m.p. 203–204°. $[\alpha]_D = -31° \pm 2°$ (chloroform).

Analysis—$C_{25}H_{33}O_4Br_3$. Calculated.   C 46.97, H 5.51, Br 37.50
Found.   " 47.12, " 5.25, " 37.51

Methyl 3,11-Diketo-12(α)-bromo-$\Delta^{1,4}$-choladienate—A solution of 3.20 gm. of methyl 3,11-diketo-2,4,12(α)-tribromocholanate in 15 ml. of freshly distilled 2,4,6-collidine was heated under a reflux in an atmosphere of carbon dioxide for 75 minutes and cooled (3). Chloroform was added and the solution was washed with water, dilute hydrochloric acid, and water. The organic phase was concentrated to dryness under reduced pressure and the residue was dissolved in methanol and treated with decolorizing carbon. Crystalline material (526 mg., m.p. 166–167°) separated slowly when the solution was concentrated. After several recrystallizations from methanol the product melted at 170.5–171.5°. The absorption spectrum of this

compound agrees with that of a 3-keto-$\Delta^{1,4}$-steroid (3). $[\alpha]_D$ = +17° ± 2° (chloroform). λ_{max}. (ethanol) 240 mμ; ϵ 13,200.

Analysis—$C_{25}H_{33}O_4Br$. Calculated, C 62.89, H 6.97; found, C 62.65, H 7.01

3,11,20 - Triketo - 4,12(α),21 - tribromopregnane—3,11,20 - Triketo-12(α),21-dibromopregnane (1.365 gm.) was dissolved in 20 ml. of acetic acid and 0.18 ml. of 2.7 N hydrogen bromide in acetic acid was added. Anhydrous sodium acetate (230 mg.) was dissolved in 5.50 ml. of 1.02 N bromine in acetic acid. The latter solution was then added to the solution of steroid as rapidly as the bromine was consumed. After addition of the bromine, water was added immediately. The solution was extracted with chloroform and the organic phase was washed with water, dilute sodium bicarbonate solution, and water, and was dried. The solution was concentrated under reduced pressure and crystals were obtained upon addition of methanol. Yield, 946 mg., m.p. 204–205°; 263 mg., m.p. 199–200°. After several crystallizations from chloroform-methanol the product melted at 200–201°. $[\alpha]_D$ = +36° ± 2° (chloroform). The melting point is dependent on the rate of heating.

Analysis—$C_{21}H_{27}O_3Br_3$. Calculated. C 44.47, H 4.80, Br 42.27
Found.   " 44.60, " 4.94, " 42.51

3,11,20 - Triketo - 4,12(α) - dibromo - 21 - acetoxypregnane—3,11,20 - Triketo-12(α)-bromo-21-acetoxypregnane (1.87 gm.) was dissolved in a warm mixture of 5 ml. of chloroform and 40 ml. of acetic acid and the solution was cooled to 25°. 2 drops of 1.0 N bromine solution in acetic acid were added and after an induction period of about 2 minutes 8.00 ml. of 1.00 N bromine solution in acetic acid were added as rapidly as it was consumed while the solution was stirred. Crystals began to separate after about two-thirds of the bromine solution had been added. Water was added and the dibromosteroid was extracted with chloroform. The chloroform solution was washed with a dilute solution of sodium thiosulfate and with water and was dried. The product (m.p. 212–215°, 91 per cent yield) separated when the chloroform was displaced with acetic acid. After recrystallization from chloroform-acetic acid the product melted at 219–220°. $[\alpha]_D$ = +39° ± 2° (chloroform).

Analysis—$C_{23}H_{30}O_5Br_2$. Calculated, C 50.56, H 5.54; found, C 50.66, H 5.71

3,11,20 - Triketo - 4 - bromo - 17(α) - hydroxy - 21 - acetoxypregnane—3,11,20-Triketo-17(α)-hydroxy-21-acetoxypregnane (808 mg.) was dissolved in 16 ml. of warm acetic acid, the solution was cooled to room temperature, and 0.40 ml. of 1.00 N hydrogen bromide in acetic acid was added. To the solution, within a period of about 1 minute, 3.85 ml. of 1.04 N

bromine in acetic acid which contained 164 mg. of sodium acetate were added dropwise. Addition of 10 ml. of water induced crystallization. About 100 ml. of water were then added and after 5 minutes the crystals were filtered off, washed with water, and dried. Yield, 858 mg. $[\alpha]_D$ = $+91° \pm 2°$ (acetone). The product was dissolved in 10 ml. of acetone, 20 ml. of ethyl acetate were added, and the solution was concentrated under reduced pressure to 7 ml. Yield, 594 mg. $[\alpha]_D$ = $+97° \pm 2°$ (acetone). Further purification from acetone-ethyl acetate gave a product with $[\alpha]_D$ = $+102° \pm 2°$ (acetone). When placed on the apparatus at 190° and the temperature increased 5° per minute, the material melted at 205–206°. This product does not depress the melting point of 3,11,20-triketo-2-bromo-17(α)-hydroxy-21-acetoxypregnane. The melting point is useless as a criterion of purity.

Analysis—C$_{23}$H$_{31}$O$_6$Br. Calculated. C 57.14, H 6.46, Br 16.53
Found. " 57.01, " 6.65, " 16.74

When the bromination was carried out without the use of sodium acetate, the specific rotation of the whole crystalline product was about the same as when sodium acetate was used. However, several recrystallizations did not raise the specific rotation to more than $+94°$.

3,11,20 - Triketo - 2 - bromo - 17(α) - hydroxy - 21 - acetoxypregnane— The aqueous filtrate from the preparation of the 4-bromo isomer described in the preceding paragraph was diluted to 300 ml. with water and extracted with two 25 ml. portions of chloroform. The chloroform solution was concentrated under reduced pressure to dryness and the residue was crystallized from acetone-carbon tetrachloride to give 48 mg. of product; $[\alpha]_D$ = $+50° \pm 2°$ (acetone). When the compound was placed on the apparatus at 190° and the temperature was increased 5° per minute, the melting point was 200–201° (decomposition). The melting point is not a satisfactory criterion of purity (see preceding paragraph). A sample was purified from acetone-carbon tetrachloride. $[\alpha]_D$ = $+51° \pm 2°$ (acetone).

Analysis—C$_{23}$H$_{31}$O$_6$Br. Calculated. C 57.14, H 6.46, Br 16.53
Found. " 57.38, " 6.58, " 16.92

3,11,20 - Triketo - 17(α) - hydroxy - 21 - acetoxypregnane—100 mg. of powdered zinc were added in portions to a solution of 97 mg. of 3,11,20-triketo-2-bromo-17(α)-hydroxy-21-acetoxypregnane in 5 ml. of acetic acid at room temperature. The mixture was shaken frequently for 30 minutes and filtered. The filtrate was concentrated under reduced pressure to dryness and distributed between chloroform and water. The aqueous phase contained 99 per cent of the theoretical amount of bromide ion. The chloroform solution was concentrated under reduced pressure to a small volume and diluted with absolute ether to give 58 mg. of crystals

that melted at 230–231° and did not depress the melting point of $3,11,20$-triketo-17(α)-hydroxy-21-acetoxypregnane (5). $[\alpha]_D = +82° \pm 2°$ (acetone).

SUMMARY

A method for bromination of 3-ketosteroids in which the concentration of hydrogen bromide is controlled is described.

With 1 mole of bromine, methyl $3,11$-diketo-12(α)-bromocholanate, $3,11,20$-triketo-12(α),21-dibromopregnane, and $3,11,20$-triketo-12(α)-bromo-21-acetoxypregnane afforded good yields of 4-bromo derivatives. With 2 moles of bromine, methyl $3,11$-diketo-12(α)-bromocholanate formed a $2,4,12(\alpha)$-tribromo compound.

Disproportionation of methyl $3,11$-diketo-4,12(α)-dibromocholanate occurred in acetic acid which contained hydrogen bromide, and from the solution the $2,4,12(\alpha)$-tribromo compound, a $2,12(\alpha)$-dibromo derivative, and the $12(\alpha)$-monobromosteroid were isolated. The $2,12(\alpha)$-dibromo derivative was obtained as a $3,3$-dimethyl acetal.

In the bromination of $3,11,20$-triketo-17(α)-hydroxy-21-acetoxypregnane, 2-bromo and 4-bromo compounds were formed in a ratio of $1:3$.

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STEROIDS DERIVED FROM BILE ACIDS

XI. PREPARATION OF 3-KETO-Δ⁴-STEROIDS*

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Dehydrobromination

Testosterone, progesterone, and the hormones of the adrenal cortex have an unsaturated ketone group in ring A of the steroid nucleus. For the partial synthesis of these compounds it is necessary to introduce the double bond at 4-5 if the starting material is a bile acid such as desoxycholic acid. In a study concerned with this step several 3-keto-4-bromosteroids were prepared.

When one of these bromosteroids (3,11-diketo-4-bromonorcholanic acid) was treated with 2,4-dinitrophenylhydrazine in acetic acid, it was noticed that the hydrazone which formed was red. Since 2,4-dinitrophenyl-hydrazones of saturated ketones are yellow and those of α,β-unsaturated ketones are orange to red, this chance observation suggested that hydrogen bromide had been eliminated and that the hydrazone of an α,β-unsaturated ketone had been formed.

In a preliminary report we have shown that, when methyl 3,11-diketo-4,12(α)-dibromocholanate (1) was treated with 2,4-dinitrophenylhydrazine, hydrogen bromide was eliminated and a Δ⁴-steroidal hydrazone was formed (2). Treatment of the hydrazone with pyruvic acid led to formation of the corresponding 3-keto-Δ⁴-steroid in good yield. This method of dehydrobromination was shown to be applicable to the synthesis of corticoadrenal hormones.

Djerassi (3) has subsequently studied the reaction of 2,4-dinitrophenyl-hydrazine with a series of 3-ketobromosteroids and found that hydrogen bromide was eliminated in all instances. The mechanism of the elimination reaction has been studied by Djerassi (3) and by us (4).

At this time we wish to report the details of the work which was the subject of our preliminary communication.

Methyl 3,11-diketo-4,12(α)-dibromocholanate (1) was used as a model compound[1] for the study of this reaction. When it was treated in acetic acid with 2,4-dinitrophenylhydrazine, an orange-red hydrazone was ob-

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[1] Throughout this paper "the model compound" will be used to designate methyl 3,11-diketo-4,12(α)-dibromocholanate.

tained in about 90 per cent yield and the filtrate contained 0.95 molar equivalent of hydrogen bromide. The hydrazone was identical with the one prepared by treatment of methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate with 2,4-dinitrophenylhydrazine.

Dehydrobromination (3, 4) through activation of the bromine at C-4 results from the influence of the hydrazone at C-3, but the bromine at C-12 cannot be influenced in a similar way because the ketone group at C-11 does not form hydrazones or derivatives with other carbonyl reagents.

In the earlier experiments it was found that the solutions of the crude Δ^4-steroidal hydrazone which had been exposed to air contained variable amounts of a hydrazone with maximal absorption in the ultraviolet region at a longer wave-length than the Δ^4-steroidal hydrazone. This indicated the presence of a diene hydrazone, and suggested that autoxidation of the Δ^4-steroidal hydrazone had occurred. Methyl 3,11-diketo-12(α)-bromo-$\Delta^{4,6}$-choladienate 3-(2,4-dinitrophenylhydrazone) was subsequently isolated from solutions of the Δ^4-steroidal hydrazone which had been aerated.

More recent experiments[2] indicate that the pure Δ^4-steroidal hydrazone in the absence of hydrogen bromide is not autoxidizable. However, if dehydrobromination is carried out in the presence of air and acetic acid, or if a solution of the Δ^4-steroidal hydrazone in acetic acid which contains hydrogen bromide is aerated, some $\Delta^{4,6}$-diene hydrazone is formed. It is probable that hydrogen bromide is oxidized by air to bromine which enters the steroid at C-2 or C-6 and is then eliminated as hydrogen bromide to give the $\Delta^{4,6}$-diene hydrazone. In all later experiments the unsaturated hydrazones were formed either in an atmosphere of carbon dioxide or in the presence of sodium acetate to neutralize hydrogen bromide.

Djerassi (3) has described the formation of a $\Delta^{4,6}$-diene hydrazone by the treatment of a 3-keto-2,4-dibromosteroid with 2,4-dinitrophenylhydrazine. In our study the treatment of methyl 3,11-diketo-2,4,12(α)-tribromocholanate (1) with 2,4-dinitrophenylhydrazine in acetic acid gave a $\Delta^{4,6}$-diene hydrazone identical with the one formed by aeration of the Δ^4-steroidal hydrazone. The 3,11-diketo-2,4,12(α)-tribromo compound formed a hydrazone much more slowly with 2,4-dinitrophenylhydrazine than did the 4,12(α)-dibromo compound. The yield was about 40 per cent under the conditions which were used.

It has been shown that methyl 3,11-diketo-12(α)-bromocholanate 3-(2,4-dinitrophenylhydrazone) can be brominated in chloroform with 1 mole of bromine to give the corresponding 4-bromohydrazone which loses hydrogen bromide when placed in acetic acid and forms the Δ^4-steroidal hydrazone (4). If, however, 2 moles of bromine are added to methyl 3,11-diketo-12(α)-bromocholanate 3-(2,4-dinitrophenylhydrazone) in chloroform and

[2] Mattox, V. R., and Kendall, E. C., unpublished data.

the dibromohydrazone is subsequently heated in acetic acid, the $\Delta^{4,6}$-diene hydrazone is obtained in 75 per cent yield. Presumably the atoms of bromine enter the steroid at C-2 and C-4 to give the same intermediate that is formed when methyl 3,11-diketo-2,4,12(α)-tribromocholanate is treated with 2,4-dinitrophenylhydrazine. Elimination of 2 moles of hydrogen bromide with rearrangement gives the $\Delta^{4,6}$-diene hydrazone.

After it had been found that methyl 3,11-diketo-4,12(α)-dibromocholanate undergoes disproportionation in acetic acid which contains hydrogen bromide (1), the hydrazone was formed in acetic acid in the presence of enough sodium acetate to neutralize the hydrogen bromide eliminated coincident with formation of the hydrazone. This procedure gave slightly better results than were obtained without the sodium acetate. With larger quantities of sodium acetate or pyridine appreciable amounts of the 4-acetoxy hydrazone were formed.

Treatment of the compound designated methyl 2,12(α)-dibromo-3,3-dimethoxy-11-ketocholanate (1) with 2,4-dinitrophenylhydrazine in acetic acid resulted in hydrolysis of the dimethyl acetal group and the separation of a product which is formulated as methyl 3,11-diketo-12(α)-bromo-Δ^1-cholenate 3-(2,4-dinitrophenylhydrazone). The position of the double bond in the steroid was shown to be at 1-2 after restoration of the keto group at C-3. Use of 2,4-dinitrophenylhydrazine for introduction of a double bond was applied by Djerassi (3) to 3-keto-2-bromosteroids and afforded Δ^1-steroidal hydrazones.

When methyl 3,11-diketo-4,12(α)-dibromocholanate was treated with 2,4-dinitrophenylhydrazine in methanol (4) a mixture of methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate 3-(2,4-dinitrophenylhydrazone) and methyl 3,11-diketo-12(α)-bromo-4-methoxycholanate 3-(2,4-dinitrophenylhydrazone) was formed. The position of the methoxyl group was shown by elimination of methanol from the hydrazone by heating for a short time in acetic acid with small amounts of perchloric or hydrobromic acid to give the Δ^4-steroidal hydrazone. The hydrazones tend to separate as salts unless the mineral acid is removed or neutralized. The elimination of water from α- or β-hydroxy carbonyl compounds upon formation of hydrazones has been recorded in several instances (5).

Experiments were performed with acetic acid as the solvent to determine whether other substituted hydrazines could be used instead of 2,4-dinitrophenylhydrazine. With phenylhydrazine the hydrogen bromide was eliminated quantitatively from the model compound and a derivative was obtained which had an ultraviolet spectrum identical with that of the phenylhydrazone prepared from methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate. However, since the phenylhydrazone was unstable, it was not investigated further. Hydrogen bromide was eliminated almost quanti-

tatively from the model compound with α,α-diphenylhydrazine, but crysstalline material was not isolated.

Girard's Reagent T caused quantitative removal of the bromine. However, after hydrolysis of the product with mineral acid to restore the 3-keto group no crystalline material was obtained.

Treatment of the model compound with semicarbazide hydrochloride (3, 4) resulted in quantitative removal of the halogen from C-4 and gave about 45 per cent of the Δ^4-steroidal semicarbazone. Both hydroxylamine (3) and hydrazine almost quantitatively removed the bromine; however, only the former reagent gave rise to a crystalline product.

A consideration of the mechanism (4) by which bromine was removed suggested that, if loss of the halogen atom at C-4 depended on formation of a RN=C— bond at C-3 and then donation of electrons to the halogen at C-4, then amines in which R— could act as an electron donor might form Schiff bases and cause loss of halogen. It was found that p-phenylenediamine, p-ethoxyaniline, p-hydroxyaniline, and aniline induced loss of some bromide ion. Other amines in which R— has little tendency to donate electrons should not affect the atom of halogen. Benzylamine (3), p-nitroaniline, and isoamylamine did not cause loss of bromide ion.

Restoration of Keto Group

With a method available for conversion of a 3-keto-4-bromosteroid to a Δ^4-steroidal hydrazone in about 85 per cent yield it was evident that if the hydrazine moiety could be removed from C-3 a method would be available for synthesis of 3-keto-Δ^4-steroids. The work of Conant and Bartlett (6) suggested that pyruvic acid might serve admirably for this purpose, since it contains a carboxyl group and its hydrazone could be separated from a neutral substance.

Cleavage of the saturated steroidal hydrazones or semicarbazones with pyruvic acid as an acceptor occurred readily. Methyl 3,11-diketo-4-methoxy-12(α)-bromocholanate was prepared both from its 3-semicarbazone (4) and its 3-(2,4-dinitrophenylhydrazone) (4).

The rate of cleavage of α,β-unsaturated steroidal hydrazones was slow unless mineral acid was used as a catalyst. When methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate 3-(2,4-dinitrophenylhydrazone) was mixed with aqueous[3] pyruvic acid in chloroform and enough hydrogen bromide in acetic acid was added to make the solution 0.1 N, the initially red solution

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[3] The pyruvic acid used was supplied by the Paragon Testing Laboratories and contained approximately 19 per cent water. We were not aware of the necessity of having water present until this was pointed out by Dr. Jacob van de Kamp of Merck and Company, Inc., and subsequently by Dr. Carl Djerassi (3).

became yellow in about 2.5 to 3 hours. The pyruvic acid 2,4-dinitro-phenylhydrazone was removed with sodium bicarbonate. Any 24-carboxyl group was reesterified with diazomethane and the ultraviolet spectrum at 387 mμ showed that not more than 1 to 2 per cent of unchanged hydrazone was present. The small amount of hydrazone was removed by adsorption on activated carbon, and colorless methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate was separated in 94 per cent yield. If the Δ^4-steroidal hydrazone was contaminated with a few per cent of $\Delta^{4,6}$-diene hydrazone, the solution remained red for many hours and it was not possible to determine visually when all of the Δ^4-steroidal hydrazone had been cleaved.

The addition of a small amount of water beyond that which the pyruvic acid contained (about 18 to 20 per cent) did not significantly alter the rate of reaction. Sodium hydroxide decreased the speed of the removal of the hydrazine with pyruvic acid. However, hydrogen bromide (0.01 to 0.10 N) increased the rate of reaction. The rate of cleavage of the hydrazone with 1 N hydrogen bromide was much slower than with 0.10 N.

Methyl 3,11-diketo-12(α)-bromo-Δ^1-cholenate 3-(2,4-dinitrophenylhy-drazone) was converted to the 3-keto-Δ^1-steroid by the same procedure used for the corresponding 3-keto-Δ^4-steroid. The maximal absorption of the 3-keto-Δ^1 compound occurred at 224 mμ. This maximum was at a point which was 6 to 7 mμ shorter than the maximum reported for other 3-keto-Δ^1-steroids (7, 8).

Hydrolysis of the $\Delta^{4,6}$-steroidal hydrazones proceeds much more slowly than does that of the corresponding Δ^4-compounds (3). This property has been utilized in the separation of a $\Delta^{4,6}$-diene hydrazone from a mixture of this material and the Δ^4-steroidal hydrazone. Reversal of the diene hydrazone then gave a preparation of the 3-keto-$\Delta^{4,6}$-diene free from the 3-keto-Δ^4-steroid. Methyl 3,11-diketo-12(α)-bromo-$\Delta^{4,6}$-choladienate was obtained from the corresponding hydrazone in about 65 per cent yield when the reaction time was extended to 23 hours.

EXPERIMENTAL

All melting points were taken on a Fisher-Johns apparatus and recorded as read. Specific rotations were taken at a concentration of approximately 1 gm. per 100 ml. of solution. The absorption spectra were determined on a Beckman model DU spectrophotometer which had been standardized against a mercury lamp. The spectra of all hydrazones were taken in chloroform, unless otherwise designated. The compounds described in this paper were analyzed by Mr. Joseph F. Alicino, Metuchen, New Jersey. and in the laboratory of Merck and Company, Inc., Rahway, New Jersey.

Methyl 3,11-Diketo-12(α)-bromo-Δ^4-cholenate 3-(2,4-Dinitrophenylhydra-

zone)—At room temperature 50 ml. of acetic acid were added to 560 mg. of methyl 3,11-diketo-4,12(α)-dibromocholanate, 90 mg. of sodium acetate, and 240 mg. of 2,4-dinitrophenylhydrazine and the mixture was shaken for a few minutes. After 3 hours 100 ml. of water were added and the solution was filtered. The filtrate contained 0.95 m.eq. of Br⁻. Two crops of crystals were obtained from benzene and a third was obtained from acetic acid. Yield 436 mg., m.p. 239–240°, λ_{max}. 388 mμ, ϵ 30,700; 92 mg., m.p. 236–237°, λ_{max}. 388 mμ, ϵ 31,500; 70 mg., m.p. 233–234°, λ_{max}. 388 mμ, ϵ 31,400.

In an experiment like the one described in the above paragraph, except that no sodium acetate was used, 9.50 ml. of 0.1 N Br⁻ were removed. Yield 262 mg., m.p. 238–239°, λ_{max}. 386 mμ, ϵ 30,700; 150 mg., m.p. 238–239°, λ_{max}. 388 mμ, ϵ 31,100; 210 mg., m.p. 238–239°, λ_{max}. 390 mμ, ϵ 31,400.

Analysis—$C_{31}H_{39}O_7BrN_4$. Calculated. C 56.45, H 5.96, Br 12.12
　　　　　　　　　　　Found.　　" 56.21, " 5.89, " 11.93

Methyl 3,11-diketo-12(α)-bromo-Δ⁴-cholenate (959 mg.) and 436 mg. of 2,4-dinitrophenylhydrazine were added to 50 ml. of acetic acid. Red crystals began to separate after 15 minutes. After 1.5 hours the solution was heated to boiling and cooled, and the red crystals (1.2 gm., m.p. 238–239°) were filtered off. After crystallization from benzene the melting point was 240–240.5°. λ_{max}. 387 mμ, ϵ 30,500.

Methyl 3,11-diketo-4-methoxy-12(α)-bromocholanate 3-(2,4-dinitro-phenylhydrazone) (69 mg.) was sealed in a tube with 2.5 ml. of 0.10 N perchloric acid in glacial acetic acid under carbon dioxide and warmed to 60°. After 30 minutes yellow crystals separated. After 4.5 hours at about 60° the solution was cooled and the crystals were filtered off and washed with chloroform. Yield 58 mg. (76 per cent), m.p. 230–235° (decomposition). This product is the perchlorate salt of the Δ⁴-steroidal hydrazone. A sample (52.9 mg.) was dissolved in 1.0 ml. of pyridine, 15 ml. of water were added, the red crystals were filtered off, and the filtrate was titrated with 0.10 N sodium hydroxide and phenolphthalein. Equivalent weight found, 750; theory, 760. The red crystals were recrystallized from acetic acid; yield 42 mg., m.p. 243–244°. There was no depression of the melting point on admixture with methyl 3,11-diketo-12(α)-bromo-Δ⁴-cholenate 3-(2,4-dinitrophenylhydrazone). λ_{max}. 388 mμ, ϵ 29,500.

The acetic acid-perchloric acid filtrate was diluted with chloroform and washed with water. Concentration of the chloroform phase and crystallization from acetic acid gave 12 mg. of material, m.p. 242–243°, λ_{max}. 387 mμ, ϵ 30,000.

The yellow perchlorate of the Δ⁴-steroidal hydrazone was prepared in

64 per cent yield from 1 mM of the Δ^4-steroidal hydrazone in 25 ml. of 0.10 N perchloric acid in acetic acid. The solution was boiled for 3 minutes. After cooling, the crystals which separated were filtered and washed with acetic acid and with dry alcohol-free chloroform. A sample of the perchlorate (480 mg.) was dissolved in 2 ml. of pyridine, 25 ml. of water were added, and the red crystals were filtered off and washed with water. Recrystallization from acetic acid gave 382 mg. of red Δ^4-steroidal hydrazone, m.p. 243–244°. Titration of the filtrate with 0.10 N sodium hydroxide and phenolphthalein indicated an equivalent weight of 762, theoretical value 760.

Aeration of Methyl 3,11-Diketo-12(α)-bromo-Δ^4-cholenate 3-(2,4-Dinitrophenylhydrazone)—40 mg. of 2,4-dinitrophenylhydrazine were dissolved in 50 ml. of acetic acid in each of two small flasks. A current of air was passed through one solution and a current of carbon dioxide through the other. After 5 minutes 112 mg. of methyl 3,11-diketo-4,12(α)-dibromocholanate were added to each solution. After 7 hours the flasks were stoppered and allowed to stand overnight. Each solution was concentrated to about 2 ml. and crystals were separated, 88 mg. from the carbonated solution, λ_{max} 387 mμ, ϵ 30,800; 86 mg. from the aerated solution, λ_{max}. 390 mμ, ϵ 33,100. The absorption spectrum of the aerated solution indicated the presence of methyl 3,11-diketo-12(α)-bromo-$\Delta^{4,6}$-choladienate 3-(2,4-dinitrophenylhydrazone) whose absorption constants are λ_{max}. 397 mμ, ϵ 36,600.

Since this work was completed, Djerassi and Ryan (9) have shown that $\Delta^{4,6}$-diene 2,4-dinitrophenylhydrazones have a second absorption maximum at 309 mμ in addition to the principal one at about 390 mμ, and that Δ^4-steroidal hydrazones have an absorption minimum at 317 mμ, ϵ 3200. The comparison of spectra in this region furnishes a more sensitive measure of the amount of $\Delta^{4,6}$-diene in Δ^4-hydrazones than it is possible to obtain in the region from 385 to 400 mμ.

Two additional experiments were performed with the same quantities of material and conditions described in the preceding paragraph except that air was passed through both solutions and one solution contained 20 mg. of sodium acetate.

From the solution which contained sodium acetate, 117 mg. of crystals were obtained. λ_{min}. 317 mμ, ϵ 3200; λ_{max}. 388 mμ, ϵ 31,100. The molecular extinction coefficient at 317 mμ indicated that this product was not contaminated with $\Delta^{4,6}$-steroidal hydrazones. From the other solution 102 mg. were obtained. At 317 mμ, ϵ 5100; λ_{max}. 391 mμ, ϵ 32,400. The molecular extinction coefficient at 317 mμ showed the presence of $\Delta^{4,6}$-steroidal hydrazone.

Methyl 3,11-Diketo-12(α)-bromo-$\Delta^{4,6}$-choladienate 3-(2,4-Dinitrophenyl-

hydrazone)—A current of air was passed through a solution of 1.65 gm. of crude methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate 3-(2,4-dinitrophenyl-hydrazone) on the steam bath for 3 hours. The solution was concentrated and crystals were obtained from acetic acid; λ_{max}. 389 mμ, ϵ 32,400. The crystals and mother liquors were combined and air was passed through the solution for 7 hours at room temperature. Crystals were obtained by concentration; 1.12 gm., λ_{max}. 393 mμ, ϵ 33,500. According to the spectrum this material contained roughly 50 per cent $\Delta^{4,6}$-diene hydrazone. The Δ^4-hydrazone was separated from the $\Delta^{4,6}$-diene hydrazone as follows: 1.1 gm. of the hydrazone and 3.8 ml. of 2.3 N hydrogen bromide in acetic acid were added to 33 ml. of chloroform and 50 ml. of 81 per cent pyruvic acid. The solution was maintained at about 45° in a stoppered flask for 3 hours. Chloroform was added and the organic phase was washed with water, repeatedly with dilute sodium bicarbonate solution until the aqueous phase was colorless, and with water. The chloroform solution was dried and evaporated almost to dryness. Addition of methanol caused separation of bright red needles (260 mg.), m.p. 245–246° (decomposition). After two crystallizations from chloroform-methanol the material melted at 248–249°. λ_{max}. 397 mμ, ϵ 36,600.

Analysis—$C_{31}H_{37}O_7BrN_4$.   Calculated.   C 56.62, H 5.67
                                   Found.   " 56.73, " 5.92

The $\Delta^{4,6}$-steroidal hydrazone was obtained in about 40 per cent yield from a solution of 320 mg. of 3,11-diketo-2,4,12(α)-tribromocholanate (1) and 396 mg. of 2,4-dinitrophenylhydrazine in 10 ml. of chloroform, 40 ml. of acetic acid, and 0.44 ml. of 2.3 N hydrogen bromide in acetic acid after 16 hours at room temperature. The hydrazone was extracted with chloroform after addition of water and was crystallized from acetic acid.

The yield of $\Delta^{4,6}$-diene hydrazone (12 per cent) obtained from the 2,4,12(α)-tribromo compound in acetic acid without addition of hydrogen bromide was unsatisfactory. Two successive treatments (10 minutes each) of the tribromo compound in acetic acid at the boiling point with 4 moles of the hydrazine yielded 35 per cent of the theoretical amount of bromide ion. Both 3-keto-2,4,12(α)-tribromosteroid and dinitrophenyl-hydrazine acetate were isolated. The $\Delta^{4,6}$-hydrazone was identified by its melting point (248–249°) and spectrum. λ_{max}. 308 mμ, ϵ 13,200; 398 mμ, ϵ 36,200.

Methyl   3,11-diketo-12(α)-bromocholanate   3-(2,4-dinitrophenylhy-drazone) (662 mg.) was dissolved in 100 ml. of dry alcohol-free chloroform, and 4.00 ml. of 1.0 N bromine in chloroform were added in one portion. After about 2 minutes all of the bromine had been consumed. After 3 minutes 100 ml. of acetic acid were added and the yellow solution was con-

centrated under reduced pressure almost to dryness. Acetic acid (25 ml.) was added and the solution was heated under reflux for 15 minutes. Red crystals formed. The solution was cooled and the crystals were collected. Yield 497 mg. (75 per cent), m.p. 251–255°; no depression of the melting point of the known $\Delta^{4,6}$-steroidal hydrazone. $\lambda_{max.}$ 309 mμ (9), ϵ 11,600; 396 mμ, ϵ 36,000.

Methyl 3,11-Diketo-12(α)-bromo-Δ^1-cholenate 3-(2,4-Dinitrophenylhydrazone)—303 mg. of methyl 2,12(α)-dibromo-3,3-dimethoxy-11-ketocholanate and 120 mg. of 2,4-dinitrophenylhydrazine were dissolved in 20 ml. of acetic acid at room temperature and the solution was sealed in an atmosphere of carbon dioxide. After 24 hours the solution was distributed between chloroform and water. The halogen ion removed = 3.7 ml. of 0.10 N (74 per cent of theoretical quantity). The organic phase was evapdrated to dryness under reduced pressure. The residue was dissolved in 1.0 ml. of acetic acid and the solution was refluxed for 5 minutes under carbon dioxide, with use of a water trap to catch any escaping hydrogen bromide. The solution was distributed between chloroform and water; 1.0 ml. of 0.10 N bromide ion was removed. The orange-colored hydrazone was crystallized from acetic acid; yield 302 mg., m.p. 205–208°. Several recrystallization raised the melting point to 212–213°. $\lambda_{max.}$ 381 mμ, ϵ 29,200(9).

Analysis—$C_{31}H_{39}O_7N_4Br$. Calculated. C 56.45, H 5.96, Br 12.12
                             Found.   " 56.45, " 6.16, " 12.11

Methyl 3,11-Diketo-12(α)-bromo-Δ^4-cholenate 3-Phenylhydrazone—Phenyl-hydrazine (13 mg.) in 1.0 ml. of acetic acid was mixed with 0.10 mM of methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate in 3.0 ml. of acetic acid. After 30 minutes at room temperature chloroform was added and the solution was washed with water, with dilute hydrochloric acid, and with water. An aliquot of the chloroform solution was diluted with methanol, $\lambda_{max.}$ 324 mμ, ϵ 23,500. 0.10 mM of methyl 3,11-diketo-4,12(α)-dibromo-cholanate was treated in a similar manner. $\lambda_{max.}$ 323 mμ, ϵ 24,600.

2 mM of methyl 3,11-diketo-4,12(α)-dibromocholanate and 261 mg. of phenylhydrazine were dissolved in 35 ml. of acetic acid at room temperature. After 18 hours the dark colored solution was distributed between benzene and water. The aqueous phase contained 1.92 m.eq. of bromide ion. Crystals (270 mg.) were obtained from methanol, but the hydrazone was unstable and was not investigated further.

Treatment of Model Compound with Girard's Reagent T—560 mg. of methyl 3,11-diketo-4,12(α)-dibromocholanate and 506 mg. of Girard's Reagent T were dissolved in 15 ml. of acetic acid at room temperature. After 16 hours 180 ml. of water and 5.5 ml. of concentrated sulfuric acid

were added. Chloroform was added after 12 hours and 83 per cent of 1 equivalent of halogen ion in addition to that contained in the Girard's reagent was found in the aqueous phase. The organic phase was concentrated under reduced pressure to dryness. Absorption in methanol at 238 mμ indicated that not more than 12 per cent of 3-keto-Δ^4-steroid was present.

Treatment of Model Compound with α,α-Diphenylhydrazine—1 mM of methyl 3,11-diketo-4,12(α)-dibromocholanate, 1.20 mM of α,α-diphenylhydrazine hydrochloride, and 2.40 mM of sodium acetate were dissolved in 25 ml. of acetic acid under an atmosphere of CO_2. After 24 hours at room temperature the mixture was very dark. The product was distributed between water and chloroform. The organic phase contained 0.97 mM of halide ion in excess of that from the diphenylhydrazine hydrochloride. It was not possible to crystallize the hydrazone.

Treatment of Model Compound with Hydrazine—1 mM of methyl 3,11-diketo-4,12(α)-dibromocholanate was dissolved in 15 ml. of acetic acid, and 308 mg. of sodium acetate, 156 mg. of hydrazine sulfate, and 2.0 ml. of water were added. After 22 hours at room temperature chloroform was added and the solution was washed with water. The aqueous phase contained 91 per cent of the theoretical amount of bromide ion. A crystalline hydrazone was not obtained.

Treatment of Model Compound with Hydroxylamine—To 1 mM of methyl 3,11-diketo-4,12(α)-dibromocholanate in 15 ml. of acetic acid were added 199 mg. of sodium acetate, 99 mg. of hydroxylamine sulfate, and 2.0 ml. of water at room temperature. After 22 hours water was added and the solution was extracted with chloroform. The aqueous phase contained 89 per cent of the theoretical amount of bromide ion. The chloroform solution was washed with dilute nitric acid and water and concentrated under reduced pressure. Crystals (123 mg., m.p. 142–144°) were obtained from methanol. The product melted at 154–156° after several recrystallizations from methanol. The maximal absorption in the ultraviolet region occurred at a longer wave-length than that reported for an unsubstituted Δ^4-steroidal oxime (10). The analysis indicated that this product was either an *O*-acetyl or *N*-acetyl derivative of methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate; however, it was not studied further. λ_{max}. (methanol) 250 mμ, ϵ 9740.

Analysis—$C_{27}H_{38}O_5NBr$. Calculated. C 60.44, H 7.14, N 2.61, CH_3CO 8.02
Found.   " 60.82, " 7.23, " 2.75, "   7.54

Treatment of Model Compound with Amines—In the experiments of Table I 1.0 mM of methyl 3,11-diketo-4,12(α)-dibromocholanate was dissolved in 15 ml. of glacial acetic acid, 2.0 mM of amine were added, and the solution was maintained under carbon dioxide for 16 hours. Water was

added and the solution was extracted with chloroform. The halogen in the aqueous phase was determined by the method of Volhard when the solution was not colored; otherwise it was precipitated as silver bromide.

Methyl 3,11-Diketo-4-methoxy-12(α)-bromocholanate—5 ml. of pyruvic acid (approximately 81 per cent) were mixed with 1.5 ml. of 5.0 N aqueous sodium hydroxide[4] with cooling and 200 mg. of methyl 3,11-diketo-4-methoxy-12(α)-bromocholanate 3-semicarbazone (4) were then added to the mixture. After 46 hours at room temperature chloroform was added and the solution was washed with water, with dilute sodium hydroxide solution, and with water. After evaporation in an air current, crystals were obtained from aqueous methanol; 118 mg., m.p. 141–142°. Upon

TABLE I

Treatment of Model Compounds with Amines

Amine	Per cent 1.0 mM bromide ion removed
p-Phenylenediamine	85
p-Hydroxyaniline	19
p-Ethoxyaniline	16
Aniline	23
p-Nitroaniline	0
Benzylamine	0
Isoamylamine	0

recrystallization from aqueous methanol the melting point was raised to 142–143°. $[\alpha]_D = +29° \pm 2°$ (chloroform).

Analysis—$C_{26}H_{39}O_5Br$. Calculated. C 61.05, H 7.69, CH_3O 12.14
Found. " 61.19, " 7.74, " 12.62

A solution of 69 mg. of methyl 3,11-diketo-4-methoxy-12(α)-bromocholanate 3-(2,4-dinitrophenylhydrazone) (4) in 4 ml. of chloroform, 2 ml. of acetic acid, and 1 ml. of 81 per cent pyruvic acid was maintained at 45° under an atmosphere of carbon dioxide for 45 hours. The solution was diluted with chloroform and water, washed with aqueous sodium bicarbonate solution and with water, and concentrated under reduced pressure to dryness. The residue was treated with activated carbon in methanol and crystallized from aqueous methanol. Yield 38 mg. (75 per cent), m.p. 140–141°; no depression on admixture with methyl 3,11-diketo-4-methoxy-12(α)-bromocholanate. $[\alpha]_D = +30° \pm 2°$ (chloroform).

Methyl 3,11-Diketo-12(α)-bromo-Δ⁴-cholenate (a)—A solution of 660 mg. of methyl 3,11-diketo-12(α)-bromo-Δ⁴-cholenate 3-(2,4-dinitrophenylhydrazone) (which had been prepared from the 3-keto-Δ⁴-steroid) in 20 ml. of

[4] Conant and Bartlett (6) have suggested pyruvic acid, 10 per cent, neutralized with sodium hydroxide for cleavage of semicarbazones.

chloroform, 30 ml. of approximately 80 per cent pyruvic acid, and 2.2 ml. of 2.3 N hydrogen bromide in acetic acid was maintained at approximately 45° for 17 hours.   (Later experiments indicated that 3 hours were ample.) Chloroform was added and the solution was washed with water, three times with dilute sodium bicarbonate solution, and with water.   Any free carboxyl group at C-24 was esterified with a solution of diazomethane in ether, and the solution was washed with water, with dilute sodium carbonate solution, and with water.   The solvent was removed under reduced pressure and crystals were obtained from methanol; yield 430 mg.   Some samples of the crystals partially melted at about 172–174°, recrystallized on the slide, and melted again at 187–188°; other samples melted at 187–188° without previous softening.   The product did not depress the melting point of methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate (1).   An additional 22 mg. were obtained from the mother liquor; m.p. 185–187° after partially melting at 172°.   The total yield was 94.5 per cent.

(b) A solution of 560 mg. of methyl 3,11-diketo-4,12(α)-dibromocholanate, 410 mg. of sodium acetate, and 240 mg. of 2,4-dinitrophenylhydrazine in 50 ml. of acetic acid was sealed in an atmosphere of carbon dioxide. After 22 hours at room temperature the solvent was removed under reduced pressure and the residue was dissolved in 20 ml. of chloroform, 30 ml. of 81 per cent pyruvic acid, and 4.0 ml. of 2.3 N hydrogen bromide in acetic acid.   The flask was sealed under carbon dioxide and placed in a constant temperature bath at 45°.   After 3 hours the product was treated as described in the previous paragraph.   Yield 310 mg., m.p. 188–189°; 43 mg., m.p. 184–186°.   Total yield, 74 per cent.

The residue from the filtrate was extracted with 40 ml. of boiling ligroin. Concentration of the solvent afforded 5 mg. of methyl 3,11-diketo-4-acetoxy-12(α)-bromocholanate which after recrystallization from methanol melted at 145–146° (after first melting at 110–112°).   It showed no selective absorption between 220 and 320 mμ (see the following paragraph).

Methyl 3,11-Diketo-4-acetoxy-12(α)-bromocholanate—A solution of 560 mg. of methyl 3,11-diketo-4,12(α)-dibromocholanate, 0.81 ml. of pyridine, and 240 mg. of 2,4-dinitrophenylhydrazine in 50 ml. of acetic acid was sealed under carbon dioxide at room temperature.   After 17 hours enough hydrogen bromide (3.52 ml. of 2.56 N) in acetic acid was added to neutralize the pyridine and the solution was concentrated under reduced pressure to dryness.   The product was treated with pyruvic acid as described under methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate (a).   The chloroform which contained the products resulting from cleavage of the hydrazone was displaced with methanol.   After removal of 10 per cent of unchanged hydrazone and 42 per cent of crude methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate

the filtrate yielded 19 per cent of methyl 3,11-diketo-4-acetoxy-12(α) bromocholanate which melted at 109–111°, recrystallized on the slide, and melted at 147–149°. A sample purified from methanol melted at 113–114° and 151–151.5°. After the compound had been dried at 100° (no weight lost), it melted at 151–151.5° without previous softening. $[\alpha]_D = +25° \pm 2°$ (chloroform).

Analysis—$C_{27}H_{39}O_6Br$. Calculated. C 60.11, H 7.29
                                    Found.   " 60.42, " 7.20

Methyl 3,11-Diketo-12(α)-bromo-Δ^1-cholenate—A solution of 208 mg. of methyl 3,11-diketo-12(α)-bromo-Δ^1-cholenate 3-(2,4-dinitrophenylhydrazone) in 6 ml. of chloroform, 9 ml. of pyruvic acid (81 per cent), and 0.37 ml. of 2.56 N hydrogen bromide in acetic acid was sealed in an atmosphere of carbon dioxide and placed in a constant temperature bath at 45°. After $3\frac{1}{4}$ hours the product was treated as described in the preparation of methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate. Two crops of crystals (126 mg., m.p. 181–183°, and 9 mg., m.p. 184–185°) were separated from methanol. A sample recrystallized from methanol melted at 186.5–187°. $[\alpha]_D = +50° \pm 2°$ (chloroform). $\lambda_{max.}$ (methanol) 224 mμ, ϵ 9890.

Analysis—$C_{25}H_{35}O_4Br$. Calculated. C 62.62, H 7.36
                                    Found.   " 62.84, " 7.39

Methyl 3,11-Diketo-12(α)-bromo-$\Delta^{4,6}$-choladienate—110 mg. of methyl 3,-11-diketo-12(α)-bromo-$\Delta^{4,6}$-choladienate   3-(2,4-dinitrophenylhydrazone) and 0.37 ml. of 2.3 N hydrogen bromide in acetic acid were added to 3.4 ml. of chloroform and 5.0 ml. of 81 per cent pyruvic acid. After 23 hours at about 45° the initially brilliant red solution had become pale orange. Chloroform and water were added, and the organic phase was washed with dilute sodium bicarbonate solution and with water. An excess of diazomethane in ether was added to esterify any free carboxyl group in the steroid. The solution was washed with dilute acetic acid, dilute sodium carbonate solution, and with water, and the solvent was removed under reduced pressure. The residue was treated with activated carbon in boiling 80 per cent methanol. Pale yellow crystals formed when the solution was concentrated; m.p. 164–165°, 55 mg. After retreatment with decolorizing carbon and two recrystallizations from aqueous methanol a colorless product was obtained; m.p. 167–167.5°, $\lambda_{max.}$ (methanol) 281 mμ, ϵ 25,900. The absorption spectrum suggests that the unsaturated system is a 3-keto-$\Delta^{4,6}$-diene (11).

Analysis—$C_{25}H_{33}O_4Br$. Calculated. C 62.88, H 6.97
                                    Found.   " 63.18, " 6.94

SUMMARY

A method is described for introduction of a double bond in 3-ketosteroids. Treatment of 3-keto-α-bromosteroids in acetic acid at room temperature with 2,4-dinitrophenylhydrazine results in dehydrobromination and formation of an α,β-unsaturated hydrazone. Removal of the hydrazine moiety with pyruvic acid affords the α,β-unsaturated ketone in good yield. Methyl 3,11-diketo-12(α)-bromo-Δ^1-cholenate and methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate have been prepared.

In the presence of even small amounts of hydrogen bromide, methyl 3,11-diketo-12(α)-bromo-Δ^4-cholenate 3-(2,4-dinitrophenylhydrazone) is converted into the hydrazone of the $\Delta^{4,6}$-diene by exposure to air. Formation of the hydrazone of the $\Delta^{4,6}$-diene is prevented by exclusion of oxygen or by addition of a base such as sodium acetate or pyridine.

The same $\Delta^{4,6}$-diene hydrazone is obtained in good yield when methyl 3,11-diketo-12(α)-bromocholanate 3-(2,4-dinitrophenylhydrazone) is brominated in chloroform with 2 moles of bromine, and the product is refluxed in acetic acid. The 3-keto-$\Delta^{4,6}$-diene can be obtained by cleavage of the hydrazone with pyruvic acid.

Methyl 3,11-diketo-4,12(α)-dibromocholanate and 2,4-dinitrophenylhydrazine in methanol give a mixture of the hydrazone of the 4-methoxy derivative and the Δ^4-steroid. In acetic acid with an excess of pyridine or sodium acetate the hydrazone of the Δ^4-steroid is formed together with some 4-acetoxy hydrazone. The 4-methoxy derivative is converted into the Δ^4-hydrazone by heating in acetic acid which contains a small amount of mineral acid.

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MIGRATION DURING HYDROLYSIS OF ESTERS OF GLYCEROPHOSPHORIC ACID

II. THE ACID AND ALKALINE HYDROLYSIS OF L-α-LECITHINS

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In a recent study (1) of the acid and alkaline hydrolysis of L-α-glycerylphosphorylcholine (L-α-GPC) it was shown that the chemical degradation of L-α-GPC is accompanied by a reversible migration of phosphoric acid and that the resulting mixtures of glycerophosphoric acids are strikingly similar in composition to those obtained from natural lecithins under comparable conditions of hydrolysis (2, 3). These observations strengthened earlier suspicions (4, 5) that a migration of phosphoric acid occurs during the hydrolysis of α-lecithins. The study of this question has hitherto been hampered by the lack of suitable lecithins, or closely related substances, in which the position of the phosphoric acid group was known. This difficulty has now been overcome by the systematic synthesis in our laboratory of the pure enantiomeric forms of several α-lecithins and of certain closely related simpler phosphoric acid esters of biological interest. By subjecting these substances (synthetic L-α-lecithins, L-α-phosphatidic acids, L-α-glycerylphosphorylcholine, and L-α-glycerophosphoric acid) to treatments customarily used in the hydrolysis of phosphatides and in determining the contents of L-α-, DL-α-, and β-glycerophosphoric acids in the hydrolysates, proof has been obtained that a reversible migration of phosphoric acid occurs during degradation of lecithins with either acid or alkali.

The nature of the intermediary compounds formed during the hydrolysis of α-lecithins has also been examined. A kinetic study has been made of the liberation of fatty acids and of choline from L-α-(dipalmitoyl)-lecithin in methanolic alkali. The observed rates of liberation suggest that both glycerylphosphorylcholine and phosphatidic acid are intermediary substances.

EXPERIMENTAL

Hydrolysis of Esters of L-α-Glycerophosphoric Acid

Substrates—The three lecithins (6, 7), L-α-(dimyristoyl)-lecithin (DML), L-α-(dipalmitoyl)-lecithin (DPL), and L-α-(distearoyl)-lecithin (DSL), as

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well as L-α-glyærylphosphorylcholine (GPC) (8) and L-α-glycerophosphoric acid (GPA) (9), were prepared synthetically as previously described. The synthesis of distearoyl-L-α-glycerophosphoric acid (DS-L-α-GPA) will soon be reported.

Alkaline Hydrolysis[1]—Solutions of 0.1 to 0.15 mM of L-α-GPA, DS-L-α-GPA, L-α-GPC, L-α-DML, L-α-DPL, and L-α-DSL in 20 ml. of 0.1 N ethanolic (95 per cent) sodium hydroxide were boiled under a reflux for 2 hours. They were then cooled, acidified with 20 ml. of 0.1 N sulfuric acid, freed of fatty acid by extraction with light petroleum (40–60°), and diluted with water. Aliquots were analyzed for total and inorganic phosphate (10) and α-GPA (11). In no case was inorganic phosphate present in significant quantities. The results are given in Table I.

TABLE I

Hydrolysis of Esters of Pure α-Glycerophosphoric Acid with Alkali

0.1 N ethanolic sodium hydroxide; 2 hours; bath temperature 100°.

Substrates		Water-soluble part of hydrolysates (50 ml.)		
L-α-	Amount hydrolyzed	Organic P per 10 ml. aliquot	Periodate consumed per 10 ml. aliquot	α-Glycerophosphate
	mg.	*mM*	*mM*	*per cent*
GPA*	43.2	0.0271	0.0272	100
DS-GPA	73.4	0.0200	0.0202	101
GPC	28.8	0.0203	0.0107	52.7
DML	76.1	0.0207	0.0119	57.5
DPL	81.1	0.0206	0.0129	62.5
DSL	87.0	0.0200	0.0106	53.0

* Anhydrous barium salt.

Acid Hydrolysis[2]—To solutions or suspensions of 0.1 to 0.15 mM of the same compounds in 0.5 ml. of 95 per cent ethanol were added 25 ml. of 6 N aqueous hydrochloric acid. The mixtures were heated at 100° for 1 hour, cooled and freed of fatty acids by extraction with 25 ml. of light petroleum (40–60°), run into 11 ml. of 12 N sodium hydroxide, and made up to 50 ml.

The total phosphate, inorganic phosphate, and α-GPA were determined as before. However, in boiling 6 N hydrochloric acid, the glycerophosphoric acid undergoes partial hydrolysis (1.5 to 3.0 per cent per hour). The consumption of periodic acid by the liberated glycerol was calculated (1 mM of inorganic P = 1 mM of glycerol = 2 mM of HIO_4) and the value

[1] An all-glass apparatus was used. A blank consisting of 20 ml. of 0.1 N ethanolic sodium hydroxide was run concurrently.

[2] The hydrolyses were carried out in an all-glass apparatus. A blank of 25 ml. of 6 N hydrochloric acid was run concurrently.

was deducted from that of the total periodic acid consumption. The corrected value was used in calculating the content of α-GPA. The data are presented in Table II

Although α-GPA on treatment with alkali remains unchanged and the phosphatidic acid under the same conditions yields almost the theoretical amount of pure α-GPA (Table I), the hydrolysates of all other α esters, including those obtained by acid treatment of α-GPA and of the α-phosphatidic acid, contain considerably less α-GPA than should be expected if no migration occurs. In the absence of significant amounts of inorganic phosphoric acid this indicates clearly a shift of phosphoric acid from the α to the β position. In order to establish with certainty the occurrence of

TABLE II

Hydrolysis of Esters of Pure α-Glycerophosphoric Acid with Acid

6 N aqueous hydrochloric acid; 1 hour; bath temperature 100°.

Substrates		Water-soluble part of hydrolysates (50 ml.)						
L-α-	Amount hydrolyzed	Total P per 10 ml. aliquot	Inorganic P per 10 ml. aliquot	Organic P per 10 ml. aliquot	Total periodate consumed	Corrected amount periodate consumed	α-GPA
	mg.	*mM*	*mM*	*mM*	*mM*	*mM*	*per cent*
GPA*............	40.5	0.0266	0.0004	0.0262	0.0229	0.0221	84.3
DS-GPA.........	89.7	0.0241	0.0008	0.0233	0.0225	0.0209	89.6
GPC............	36.2	0.0265	0.0004	0.0261	0.0224	0.0216	82.8
DML............	94.0	0.0273	0.0005	0.0268	0.0236	0.0226	84.3
DPL............	101.5	0.0271	0.0005	0.0266	0.0235	0.0225	84.6
DSL............	106.8	0.0259	0.0005	0.0254	0.0229	0.0219	86.2

* Anhydrous barium salt.

this shift and its reverse, the mixtures of glycerophosphoric acids were isolated and analyzed for their contents of the L-α, DL-α, and β isomers.

Hydrolysis with Baryta—Mixtures of 1.5 to 1.7 mM of L-α-DPL, L-α-DSL, and DS-L-α-GPA and 30 ml. of 0.55 N barium hydroxide (saturated aqueous solution at 24°) were heated with refluxing for 3 hours (oil bath temperature 110–115°). When cold, the mixtures were filtered with suction. The barium soaps were resuspended in 25 ml. of water, filtered, and washed on the filter with 25 ml. of water. The combined filtrates were freed from excess barium ions with carbon dioxide and concentrated *in vacuo* to about 10 ml. The glycerophosphoric acids were isolated as barium salt mixtures, according to the method of Folch (5).

The vacuum-dried barium salts (0.1 mm., 100°, P_2O_5, 1 hour) were analyzed for organic phosphorus, α-GPA, and optical activity (in 2 N HCl; *c*, 12). The content of L-α-GPA was found by comparing the specific

rotation of these salts with that of the barium salt of optically pure L-α-GPA ($[\alpha]_D$ = $-1.2°$),[3] prepared according to the simplified procedure of Baer and Fischer (9). Per cent L-α-GPA = $[\alpha]_D$ of sample $\times$ 100/$-1.2°$. The contents of DL-α- and β-GPA were calculated by means of the following equations: per cent DL-α-GPA = per cent total α-GPA $-$ per cent L-α-GPA; per cent β-GPA = 100 $-$ per cent total α-GPA (see Table III).

Hydrolysis with Hydrochloric Acid—To solutions of 1.5 to 1.7 mM of L-α-DPL, L-α-DSL, and DS-L-α-GPA in 2 ml. of 95 per cent ethanol and 0.5 ml. of chloroform were added 28 ml. of 1 N hydrochloric acid. The mixtures were heated with refluxing (bath temperature 110°) for 7 hours, cooled, diluted with 15 ml. of water, and extracted with 40 ml. of light petroleum (35–60°) containing 5 per cent of chloroform. The petroleum extracts were washed twice with 10 ml. of water. The aqueous-alcoholic phase and wash water were combined (approximately 65 ml.) and the gly-

TABLE III

Analysis of Glycerophosphoric Acid Mixtures Resulting from Hydrolysis of Pure
α-Phosphatides with Alkali

0.55 N aqueous baryta solution; 3 hours; bath temperature 110–115°.

Substrates		Barium glycerophosphate mixtures						
L-α-	Amount hydrolyzed	Recovery	Purity (based on P determination)	$[\alpha]_D$	Total α isomer	L-α isomer	DL-α isomer	β isomer
	gm.	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*
DPL.......	1.25	68	95.8	-0.37	46	31	15	54
DSL........	1.35	60	99.3	-0.43	46	36	10	54
DS-GPA.....	1.06	55	99.5	-1.1	97	94	3	3

cerophosphoric acids were isolated as the barium salt mixture as described previously (1). The dried barium salts were analyzed for organic phosphorus, total α-GPA, L-α-GPA, DL-α-GPA, and β-GPA. The results are given in Table IV.

The analysis of the barium glycerophosphate mixtures obtained by either acid or alkaline hydrolysis of the lecithins revealed that they contained, in addition to L-α-GPA, considerable amounts of DL-α- and β-GPA (Tables III and IV). The formation of these two compounds proves conclusively that the degradation of α-lecithins by acid or alkali involves a reversible migration of phosphoric acid ($\alpha \rightleftharpoons \beta$). Because of this migration, the elucidation of the structure and the configuration of lecithins cannot be accomplished by the use of acid or alkaline degradation procedures. At

[3] The specific rotation of L-α-GPA in 2 N hydrochloric acid was reinvestigated and found to be slightly lower than the value ($-1.45°$) reported previously (9).

present we are therefore without a reliable chemical method by means of which the constitution and configuration of an unknown lecithin can be directly established.

The barium glycerophosphate mixtures obtained by acid or alkaline hydrolysis of pure L-α-lecithins or of L-α-GPC contain α- and β-GPA in the same ratios as those obtained from natural lecithins under comparable conditions of hydrolysis (2, 3, 12); *i.e.*, approximately equal amounts of the α and β isomers on alkaline hydrolysis or predominantly the α isomer on acid hydrolysis. Furthermore, the α-GPA obtained by the alkaline hydrolysis of synthetic L-α-lecithins and of natural lecithins (3) is racemized practically to the same extent (20 to 30 per cent). It is apparent that pure synthetic L-α-lecithins and natural lecithins yield essentially the same mixture of glycerophosphoric acids, thus making it reasonably certain that the greater part, if not all, of the β-GPA in the hydrolysates of natural lecithins

TABLE IV

Analysis of Glycerophosphoric Acid Mixtures Resulting from Hydrolysis of Pure α-Phosphatides

1 N hydrochloric acid; 7 hours; bath temperature 110°.

Substrates				Barium glycerophosphate mixtures				
L-α-	Amount hydrolyzed	Recovery	Purity (based on P determination)	$[\alpha]_D$	Total α isomer	L-α isomer	DL-α isomer	β isomer
	gm.	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*
DPL........	1.21	57	99.2	−0.2	78	16	62	22
DSL.........	1.33	58	100	−0.1	84	8	76	16
DS-GPA....	1.13	62	94	−0.1	84	8	76	16

had been formed by phosphoric acid migration and not, as has been assumed, by hydrolysis of β-lecithins. It would seem, as a logical corollary, that unless other evidence is forthcoming *the natural existence of β-lecithins is unproved.*

The natural occurrence of L-α-lecithins,[4] as shown in our previous paper (1), is firmly established. There is at present no evidence indicating the natural existence of D-α- or DL-α-lecithins. The presence of DL-α-GPA in the hydrolysates of natural lecithins may be accounted for by the migration of phosphoric acid from the α to the β position, and back to α.

Among the substances studied in this paper, the DS-L-α-GPA has a somewhat exceptional position. While its acid hydrolysis follows the general pattern and yields a mixture of L-α-, DL-α-, and β-GPA (Tables II and

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[4] With regard to the stereochemical classification of the α-lecithins see Baer and Kates (6, 7).

IV), its alkaline hydrolysis produces almost pure L-α-GPA (Tables I and III). This behavior is doubtless shared by other phosphatidic acids and should permit the elucidation of their constitution and configuration by purely chemical means, e.g., alkaline degradation to GPA. It is of interest that Rae (12) has reported that GPA obtained by alkaline hydrolysis of a natural phosphatidic acid (cabbage) consists predominantly of the α form. However, no investigation of its optical activity was made.

Although the foregoing experiments establish the nature of the end-products of the acid and alkaline hydrolysis of α-lecithins, the mechanism of their formation is still conjectural. The following section describes attempts to establish the identity of the intermediary compounds in the alkaline hydrolysis and the order of their formation.

Intermediary Products in Alkaline Hydrolysis of α-Lecithins

Kinetic Study of Alkaline Hydrolysis of L-α-*(Dipalmitoyl)-lecithin*—In a 100 ml. volumetric flask was prepared a solution of 1.5 gm. of L-α-DPL in a solvent composed of 8 ml. of carbon tetrachloride and 50 ml. of absolute methanol. The solution was diluted with 9 ml. of water and placed in a bath at 37°. After 10 minutes, 20.0 ml. of a 0.98 N methanolic (95 per cent) sodium hydroxide solution were added rapidly and the solution was made up to volume with absolute methanol. The initial concentrations of the substrate and of the alkali were 0.02 M and 0.2 N respectively. The solvent consisted of 10 per cent of water, 8 per cent of carbon tetrachloride and 82 per cent of methanol (by volume). The carbon tetrachloride was added to keep the solution homogeneous throughout the experiment.

At intervals, a 5.0 ml. aliquot of the solution was withdrawn and run into a 50 ml. separatory funnel containing 5 ml. of aqueous 0.25 N sulfuric acid. The mixture was extracted twice with 20 ml. portions of light petroleum (40–60°) and the combined extracts were washed with 3 ml. of water.

To the combined aqueous layer and washings were added gradually 5 to 6 ml. of a freshly prepared 2 per cent solution of ammonium reineckate in 1.2 N hydrochloric acid and the choline reineckate was determined colorimetrically as described by Entenman, Taurog, and Chaikoff (13).

The combined petroleum ether solutions were concentrated to dryness at 40–60° in a stream of air. The residue of palmitic acid was dried *in vacuo* and titrated according to the method of Stetten and Grail (14) with thymol blue as indicator.

The total choline and palmitic acid content was determined as follows: A 5.0 ml. aliquot, made strongly alkaline with 0.5 ml. of 10 N sodium hydroxide, was boiled with refluxing for 20 minutes; to the hydrolysate, while still hot, was added 0.5 ml. of 10 N sulfuric acid and the hot mixture was

transferred quantitatively to a separatory funnel containing 5 ml. of 0.25
N sulfuric acid. The choline and palmitic acid were separated and deter-
mined as before.

Attempts to isolate the glycerophosphoric acids at the end of the hy-
drolysis (48 hours) by the lead salt method were unsuccessful, although the
water-soluble portion of the hydrolysate contained 94 per cent of the
organic phosphorus, 96 per cent of the choline, and consumed periodic acid
equivalent to 66 per cent of α-GPA.

The course of the hydrolysis of L-α-DPL, expressed in per cent of liber-
ated choline and palmitic acid, is shown in Fig. 1, Curves I and II. The
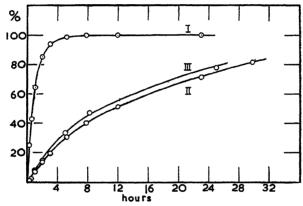
FIG. 1. Hydrolysis in 0.2 N methanolic sodium hydroxide at 37°. Liberation of
palmitic acid from L-α-DPL (Curve I) expressed in percentage of total palmitic acid.
Liberation of choline from L-α-DPL (Curve II) and from L-α-GPC (Curve III) ex-
pressed in percentage of total choline.

data yield first order reaction constants[5] averaging 6.9×10^{-2} hr.$^{-1}$ and
1.0 hr.$^{-1}$ for the liberation of choline and palmitic acid respectively.

The considerably higher rate of liberation of the fatty acids in alkaline
solution suggests that GPC is the main intermediary compound and that
practically all, and certainly not less than 75 per cent, of the free choline is
formed by hydrolysis of GPC rather than of the intact lecithin. If this is
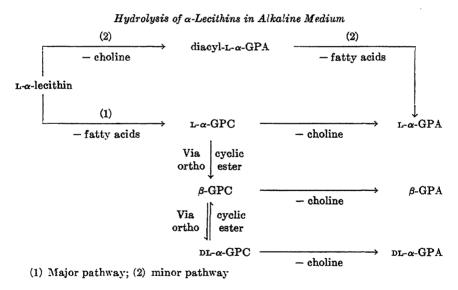
[5] Calculated according to

$$k = 2.3 \log \frac{1}{1 - \dfrac{c}{c_T}} \times \frac{1}{t \text{ (hrs.)}}$$

where c is the amount of choline or palmitic acid liberated at time t hours and c_T is
the total content of choline or palmitic acid.

the case, the rate of liberation of choline from L-α-GPC should resemble that observed in the hydrolysis of L-α-DPL. Consequently, L-α-GPC, in the presence of palmitic acid (molecular ratio of 1:2), was subjected to the same conditions of hydrolysis and the rate of liberation of choline was determined as described above. The results are shown in Fig. 1, Curve III. The curve for the liberation of choline closely resembles that for the hydrolysis of DPL. This firmly establishes GPC as the main intermediary compound in the alkaline hydrolysis of α-DPL or, in more general terms, of α-lecithins.

In view of the fact that neither the phosphatidic acids nor the glycerophosphoric acids isomerize in alkali, whereas, as we have shown earlier (1), GPC does so very readily (via its cyclic ortho ester), it is obvious that reversible migration of phosphoric acid, accompanying the alkaline hydrolysis of α-lecithins, must take place in the intermediary compounds, *i.e.* glycerylphosphorylcholine. The reaction mechanism of the alkaline hydrolysis of α-lecithins is summarized in the accompanying reaction scheme.

Hydrolysis of α-Lecithins in Alkaline Medium

(1) Major pathway; (2) minor pathway

SUMMARY

1. The acid and alkaline hydrolysis of α-lecithins is accompanied by a reversible migration of phosphoric acid which results in the formation of mixtures of α- and β-glycerophosphoric acids. These mixtures contain predominantly the α isomer when obtained by acid hydrolysis, and approximately equal amounts of the α and β isomers when obtained by alka-

line hydrolysis. The hydrolysis of the optically active forms of α-lecithins yields α-glycerophosphoric acid in partially racemized form.
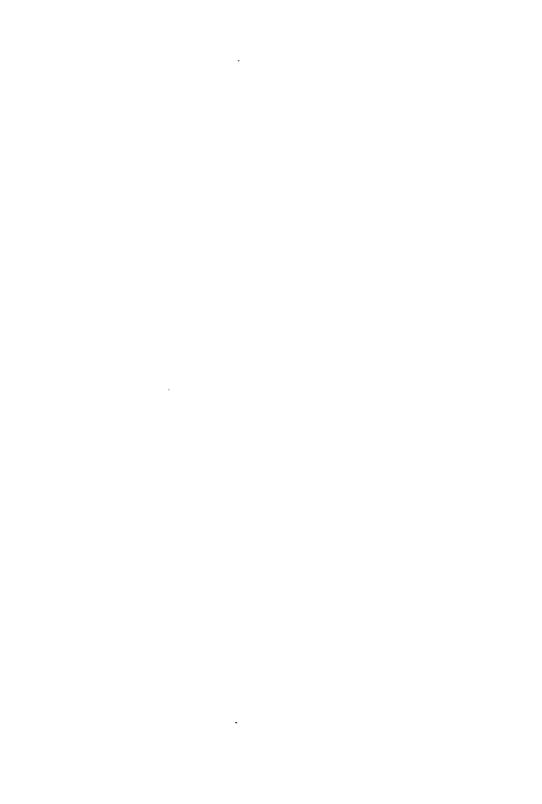
2. α-Phosphatidic acids and α-glycerophosphoric acid do not undergo migratory changes in alkaline solution but do so in acid to the same extent as the α-lecithins and α-glycerylphosphorylcholine.

3. The rates of liberation of choline and of fatty acid from a synthetic α-lecithin in alkaline solution suggest that in alkali the hydrolysis proceeds mainly via the glycerylphosphorylcholine and to a lesser extent by way of the phosphatidic acid.

4. A reaction scheme describing the hydrolysis of α-lecithins in alkaline solution is presented.

5. The migrations described invalidate the evidence generally accepted as proof for the occurrence of β-lecithins in nature.

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A STUDY OF THE URINARY EXCRETION OF α-AMINO NITROGEN AND LYSINE BY HUMANS

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In the course of experiments on the urinary excretion of lysine we have had occasion to determine α-amino nitrogen both before and after acid hydrolysis. Although the precise determination of α-amino nitrogen in urine by the manometric ninhydrin method of Van Slyke *et al.* (1) has become a fairly common clinical measurement, few data on either "free" or "total" α-amino nitrogen excretion are found in the literature, and, to our knowledge, no sex difference has been reported.

EXPERIMENTAL

24 hour urine samples were obtained from twenty-four normally healthy individuals, thirteen males and eleven females. The diet was not controlled. The samples were preserved under toluene and refrigerated. Aliquots were hydrolyzed by autoclaving overnight at 15 pounds steam pressure with 10 per cent hydrochloric acid. Hydrolyzed samples were evaporated *in vacuo* to minimize salt effects in the microbiological determination of lysine. α-Amino nitrogen was determined in the hydrolyzed samples and in samples of unhydrolyzed urine after destruction of urea by incubation with urease. The manometric ninhydrin method of Van Slyke *et al.* (1) was employed. Lysine was determined in most of the hydrolyzed samples, by use of the organism *Streptococcus faecalis* according to the method of Stokes *et al.* (2). The statistically summarized results of these determinations are shown in Table I. "Bound" α-amino nitrogen as recorded is the difference between "total" α-amino nitrogen values obtained on the hydrolyzed samples and the "free" α-amino nitrogen as determined on the unhydrolyzed samples.

DISCUSSION

In an earlier study by Thompson and Kirby (3), seven serial urine samples from each of five subjects were assayed for four amino acids: lysine, threonine, leucine, and arginine. The diet was controlled during the collection of two of the samples from each subject. Analyses on the various samples from each subject were quite uniform and unaffected by dietary control, whereas differences between subjects were in many

cases quite marked. Particularly in the case of lysine there was a definite suggestion of a sex difference, although the number of subjects was too limited to be significant. The present investigation was designed to determine whether such a sex difference actually exists, particularly in the case of lysine excretion and also for amino acids in general.

In Table I, it will be seen that the mean excretion of α-amino nitrogen, whether "total," "free," or "bound," is higher for females than for males. When expressed in mg. per day, this difference is in no case statistically significant at the 10 per cent level. If, however, the data are expressed in mg. per day per kilo of body weight, which would seem to be a more

TABLE I

Urinary Excretion of α-Amino Nitrogen and Lysine

Fraction	Expressed as	Sex	No. of sub-jects	Mean ± s.d.	t	P	Correlation with body weight values of r
Total α-amino N	Mg. per day	M.	13	251 ± 66	1.63	>0.1	+0.519
		F.	11	321 ± 138		<0.2	+0.076
	" " "	M.	13	3.48 ± 0.82	3.07	<0.01	
	per kg.	F.	11	5.90 ± 2.67			
Free α-amino N	Mg. per day	M.	13	101 ± 39	1.15	>0.2	+0.588
		F.	11	126 ± 67		<0.3	+0.095
	" " "	M.	13	1.39 ± 0.45	2.49	>0.02	
	per kg.	F.	11	2.26 ± 1.17		<0.05	
Bound α-amino N	Mg. per day	M.	13	148 ± 48	1.55	>0.1	+0.243
		F.	11	195 ± 98		<0.2	+0.043
	" " "	M.	13	2.08 ± 0.70	2.69	>0.01	
	per kg.	F.	11	3.55 ± 1.82		<0.02	
Total lysine	Mg. per day	M.	9	99 ± 41			+0.637
		F.	7	96 ± 33			+0.654
	" " "	M.	9	1.34 ± 0.47	1.62	>0.1	
	per kg.	F.	7	1.69 ± 0.49		<0.2	

For further description of the statistical methods employed see Snedecor (11).

reasonable basis of comparison, the statistical significance of the difference in means is increased in each case, the difference in total α-amino nitrogen being significant at the 1 per cent level, free α-amino nitrogen at the 5 per cent level, and bound α-amino nitrogen at the 2 per cent level. It may also be noted that the amount of urinary α-amino nitrogen excreted by females is much more variable than in the case of males, as evidenced by the larger values (in per cent) for the standard deviation from the mean.

Further evidence for a sex difference is furnished by a study of the correlation between body weight and excretion of α-amino nitrogen. Values

for the correlation coefficients as shown in Table I indicate a much more significant correlation in the case of the male samples than in that of the female samples. No statistical significance can be attached to the small positive values of r calculated for the female samples. On the other hand, the value of $+0.588$ for free α-amino nitrogen *versus* body weight for males is significant at the 5 per cent level and the value of $+0.519$ for total α-amino nitrogen *versus* body weight for males just falls short of significance at the 5 per cent level.

The data for free α-amino nitrogen seem to be in reasonable agreement with those published by Van Slyke *et al.* (1) which are given only in terms of mg. per liter. Albanese and Irby (4), using their copper method, obtained a daily excretion of 221 to 696 mg. of free α-amino nitrogen. More recently Albanese *et al.* (5) have reported considerably lower average values of 179 ± 56 and 588 ± 217 mg. per day for free and total α-amino nitrogen, respectively. These values are considerably higher than those obtained in the present investigation.

It must be recognized, of course, that total α-amino nitrogen determined on acid-hydrolyzed samples will be somewhat lower than the true values, owing to the partial or total destruction of certain amino acids, and results obtained in different laboratories may vary considerably, depending on the severity of the hydrolysis procedure. Results obtained on different urines by the same hydrolysis procedure should, nevertheless, be comparable.

No marked sex difference was noted in the urinary excretion of total lysine. The mean for female subjects when expressed as mg. per day per kilo of body weight was higher than that for males, but the difference was not significant at the 10 per cent level. Both male and female subjects exhibited a high degree of correlation between total lysine excretion and body weight.

The average lysine excretion of 97 mg. per day compares with an average obtained by Woodson *et al.* (6) of 73.2 mg. per day, based on single 24 hour samples from eighteen subjects. Results obtained from smaller numbers of subjects by Dunn *et al.* (7), by Thompson and Kirby (3), and by Scheffner *et al.* (8) are also in satisfactory agreement with the present data for lysine. Harvey and Horwitt (9) have recently reported a daily average excretion of only 16.8 mg. per day for twenty mental patients. These patients were maintained on a restricted protein intake and the data are therefore not strictly comparable.

It seems quite unlikely that the observed differences between male and female subjects could be attributed to differences in diet. The earlier study of Thompson and Kirby (3) covering four amino acids indicated no significant variation in urinary excretion with change in diet, and a more

recent report by Kirsner *et al.* (10) indicates no correlation between urinary excretion and diet in the case of six out of eight amino acids studied.

It is possible that the higher rate of amino acid excretion observed in females might be correlated with the sexual cycle, although no evidence of this was observed in the case of the four amino acids studied by Thompson and Kirby (3), when samples from the same subject were taken at various stages of the menstrual cycle. In any case, the fact remains that, as clinical determinations of urinary α-amino nitrogen are usually performed without dietary control and without reference to the menstrual cycle, one can expect significantly higher values for females than for males when results are expressed in terms of α-amino nitrogen per day per kilo of body weight.

SUMMARY

Analysis on 24 hour urine samples from twenty-four human subjects, thirteen male and eleven female, indicate a significant sex difference in the excretion of α-amino nitrogen when expressed as mg. per day per kilo of body weight. This difference is noted in both "free" and "bound" α-amino nitrogen. The amounts of α-amino nitrogen excreted by the male subjects showed a significant correlation with body weight. Such correlation was not observed with the female subjects. Data are also presented on the urinary excretion of "total" lysine by nine males and seven females.

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CATALYSIS OF TRANSAMIDATION REACTIONS BY PROTEOLYTIC ENZYMES*

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In a previous communication from this laboratory (1), evidence was presented for the view that proteinases such as papain catalyze the replacement of one participant in a peptide bond by another, closely related, molecular species (2). Replacement reactions of this type may be termed transamidation or transpeptidation reactions. The experiments to be described in what follows are concerned with the enzymatic catalysis of transamidation reactions involving the replacement of the —NH₂ group which participates in the amide bond of acylamino acid amides. Studies of replacement reactions involving peptide bonds which link two amino acid residues (transpeptidation reactions) are in progress, and will be reported in a future communication.

The data presented previously (1) dealt with the catalysis, by cysteine-activated papain, of the replacement of the amide N of benzoylglycinamide (or of carbobenzoxy-L-methioninamide) by N^{15}, introduced into the reaction mixture as isotopic diammonium hydrogen citrate. When papain was allowed to act on the amide at pH 5 until an appreciable fraction of the substrate had been hydrolyzed, the residual amide was found to contain a significant quantity of N^{15}. The extent of isotope incorporation found was considerably greater than that to be expected by direct synthesis from the hydrolytic products (acylamino acid and ammonia). It was concluded, therefore, that papain had catalyzed a transamidation reaction via a hypothetical transient intermediate in which two —NH₂ groups are attached to the carbon atom of the amide bond.

$$\text{RCO--NH}_2 \; + \; N^{15}H_3 \; \rightleftharpoons \; \left[\begin{array}{c} \text{OH} \\ | \\ \text{RC--NH}_2 \\ | \\ N^{15}H_2 \end{array} \right] \; \rightleftharpoons \; \text{RCO--}N^{15}H_2 \; + \; \text{NH}_3$$

$$R = C_6H_5CO\text{--NHCH}_2\text{--}.$$

In order to examine further the mechanism of this enzyme-catalyzed transamidation, a study has been made of the effect of pH on the extent of isotope incorporation and its relationship to the extent of hydrolysis. For this purpose, it was necessary to modify the conditions employed in the earlier studies (1). Citrate-phosphate buffers were used in place of the citrate buffer, and the cysteine concentration was increased. The latter modification was based on the finding that at pH 7 to 8 the action of

TABLE I

Catalysis of Transamidation by Papain

Initial concentration of benzoylglycinamide, 0.05 M; initial concentration of isotopic NH₄NO₃ (63.8 atom per cent excess N¹⁵ in NH₄⁺), 0.05 M; enzyme concentration, 0.26 mg. of protein N per cc. of test solution; initial concentration of activator (cysteine), 0.01 M; 0.3 cc. of methanol per cc. of test solution.

pH	Hydrolysis of amide bond*		Isolated amide†			Replacement‡
			N content	N¹⁵ concentration		
				Compound	Amide N	
	hrs.	*per cent*	*per cent*	*atom per cent excess*	*atom per cent excess*	*per cent*
5.9	6	42	15.68	0.023	0.046	0.07
7.3	6	39	15.78	0.260	0.520§	0.82
7.9	2	11	15.63	0.186	0.372	0.60
7.9	4	20	15.69	0.438	0.876	1.4
7.9	6	33	15.69	0.797	1.594	2.5
7.9	8	41	15.77	1.127	2.254	3.5
7.2‖	6	0	15.69	0.001	0.002	0.003

* Determined by measurement of ammonia liberation in Conway vessels.

† Benzoylglycinamide (theory, 15.72 per cent N) was isolated as described previously (1), and recrystallized to constant isotope concentration.

‡ $\dfrac{\text{Isotope concentration of amide N}}{\text{Isotope concentration of NH}_4{}^+ \text{ added}} \times 100.$

§ Deamidation of a sample of this preparation in a large Conway vessel (3) gave ammonia containing 0.511 atom per cent excess N¹⁵.

‖ No enzyme present.

papain requires the presence of higher cysteine concentrations than those usually employed at pH 5; this is presumably due, in large part, to the more rapid oxidation of sulfhydryl groups at alkaline pH values. An additional modification in the procedure previously described (1) was the use of isotopic ammonium nitrate (63.8 atom per cent excess N¹⁵ in the NH₄⁺ N) instead of ammonium citrate of lower isotope content.

As is shown in Table I, repetition of the studies described earlier (1) under the altered experimental conditions gave results that are quali-

tatively in accord with the earlier data. In all cases in which enzymatic hydrolysis had occurred, the residual amide was found to contain N^{15}. What is more, when the pH was raised from 5.9 to 7.9, the extent of isotope incorporation at the end of a 6 hour incubation period was markedly increased (about 36-fold), while the extent of hydrolysis during the same period decreased slightly with increasing pH. It may be concluded, therefore, that under the conditions of these experiments a change in pH from 5 to 8 has a much more profound effect on the enzyme-catalyzed transamidation reaction than on the enzymatic hydrolysis. The data in Table I suggest that it is the NH_3 molecule which reacts with the "activated" carbonyl group of the amide, since, in going from pH 5.9 to 7.9, there is approximately a 100-fold increase in the NH_3 concentration.

The data presented previously (1) indicated that, under a given set of experimental conditions, there was a proportionality between the extent of enzymatic hydrolysis and the extent of enzymatic transamidation. In order to explore this possibility further, four experiments were performed at pH 7.9, at which the greatest isotope incorporation was noted, and each of the enzyme-catalyzed reactions was allowed to proceed until a different fraction of benzoylglycinamide had been hydrolyzed. The residual amide was isolated as before, and, as will be seen from the appropriate data in Table I, a roughly proportional relationship between the extent of hydrolysis and the extent of transamidation was found, within the precision of the experimental methods employed. This result supports the suggestion made earlier (1) that the mechanism whereby the proteinase catalyzes the hydrolytic reaction also applies to the catalysis of the transamidation reaction. The enzyme may be considered to "activate" the carbonyl group of the sensitive amide bond, and a number of molecular species (*e.g.*, NH_3, H_2O, etc.) may compete for reaction with this activated group. At any given pH, a simple quantitative relationship between the extent of hydrolysis and the extent of transamidation would then be expected. However, with changes in the pH of the solution, or in the molar proportion of the competing reactants, the magnitude of the proportionality factor should change either in favor of hydrolysis or of transamidation.

It will be seen from Table I that the hydrolysis of 41 per cent of the benzoylglycinamide at pH 7.9 is accompanied by a transamidation leading to the incorporation of 2.254 atom per cent excess N^{15} into the amide N of the residual substrate. Under the conditions of these experiments, it may be expected that, as hydrolysis approaches completion, the isotope content of the residual amide will approach (but not necessarily attain) the theoretical maximum of 31.9 atom per cent excess N^{15} (*i.e.*, 50 per cent replacement). The isotope content actually found after 41 per

cent hydrolysis at pH 7.9 thus represents approximately 7 per cent transamidation. This result attests to the importance of the transamidation reaction in the system under study. What is more, the value of 7 per cent must be considered as a minimum figure for the actual extent of transamidation during the incubation period; clearly, some of the newly formed isotopic amide must have undergone hydrolysis during this period.

In extending the present studies on enzyme-catalyzed transamidation reactions, attention was drawn to the possibility that proteinases may catalyze the replacement of a participant in a peptide or amide bond by the —NHOH group of hydroxylamine.

$$RCO-NHR' + NH_2OH \rightleftharpoons RCO-NHOH + NH_2R'$$

It has long been known that reactive carbonyl groups of acylating agents readily react with hydroxylamine to give hydroxamic acids (4), which form red coordination compounds with ferric ions. These reactions have provided the basis for a colorimetric method which has been applied with signal success by Lipmann and Tuttle (5) in their studies on acetyl phosphate, an acetylating agent comparable to acetic anhydride. More recently, these authors (6) have shown that esterases and lipases present in crude tissue extracts may catalyze hydroxamic acid formation from suitable fatty acids. Chantrenne (7) had previously pointed out that the hydroxamic acid method, under a variety of experimental conditions, may not be specific for the determination of acyl phosphates.

In the experiments reported in the present communication, acylamino acid amides known to be substrates for papain were found to form hydroxamic acids on incubation with hydroxylamine in the presence of a cysteine-activated enzyme preparation. In the conduct of these experiments, aliquots of each incubation mixture were removed after various time intervals for ammonia determinations in Conway vessels. These analyses served as a measure of the quantity of the amide that had undergone hydrolysis plus that which had reacted with hydroxylamine. Separate aliquots were withdrawn for the colorimetric estimation of the hydroxamic acid present at the end of a given incubation period. In order to translate the colorimetric readings into molar quantities of the appropriate hydroxamic acid, it was desirable to have separate calibration curves for each of the hydroxamic acids to be expected in the transamidation reactions under study. In view of the number and variety of the acylamino acid amides employed, however, it was decided to assume, as a first approximation, that equimolar quantities of the corresponding hydroxamic acids would give the same intensity of color with ferric chloride under the conditions of the analytical procedure used in these experiments. Accordingly, benzoyl-L-alanylhydroxamic acid was chosen as the standard

reference substance; it was prepared by the reaction of the corresponding methyl ester with hydroxylamine. The calibration curve for the ferric salt of this hydroxamic acid is presented in Fig. 1. For comparison, the calibration curves for authentic samples of benzoylglycylhydroxamic acid and of acethydroxamic acid are also given, and it will be seen that the color values of equimolar quantities of the three compounds are quite similar. On the other hand, the slope of the curve for benzhydroxamic acid is considerably steeper (7).

Data are presented in Tables II and III on the catalysis, by cysteine-activated papain, of the replacement of the amide —NH₂ group of acyl-amino acid amides by hydroxylamine. In confirmation of earlier stud-
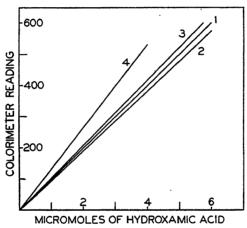
FIG. 1. Klett-Summerson calibration curves for ferric salts of hydroxamic acids. Curve 1, benzoyl-L-alanylhydroxamic acid; Curve 2, benzoylglycylhydroxamic acid; Curve 3, acethydroxamic acid; Curve 4, benzhydroxamic acid.

ies (8), it was found that hydroxylamine acts as an inhibitor of the proteolytic action of papain; when the cysteine concentration is increased, however, this inhibition can be largely overcome. A similar effect of cysteine in counteracting the inhibition of papain by the aldehyde reagent phenylhydrazine has been reported previously (9).

The data in Table II show that, as in the experiments on isotope incorporation presented above, transamidation is favored by a shift in pH from 5 to 7.5. Thus, although the rate of hydrolysis by papain is, in general, decreased at the more alkaline pH value (10), the extent of hydroxamic acid formation is markedly increased. This result has been found to apply to the action of papain on several of its synthetic substrates, i.e., benzoyl-L-argininamide, carbobenzoxy-L-isoglutamine, ben-

<div align="center">

TABLE II

Catalysis of Hydroxamic Acid Formation by Papain

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Concentration of substrates, 0.05 M; concentration of NH₂OH·HCl (adjusted to pH 6 with NaOH), 0.05 M; enzyme concentration, 0.25 mg. of protein N per cc. of test solution; cysteine concentration, 0.025 M.

Substrate	pH	Time	Ammonia liberation	Hydroxamic acid present
		min.	*μM per cc.*	*μM per cc.*
Benzoyl-L-argininamide............	4.9	20	17	0.5
		60	31	0.7
"	6.8	60	15	2.9
		120	22	3.4
"	7.5	20	7	1.35
		60	17	3.0
		120	34	4.3
"	7.2*	60	0	0.05
"	7.5†	60	0	0.1
Benzoyl-L-arginine...............	7.5	120	0	0.06
Carbobenzoxy-L-isoglutamine......	4.9	60	21	0.7
"	7.3	60	11	1.4
		120	24	2.2
"	7.2*	180	0	0.01
Carbobenzoxy-L-glutamic acid.....	7.5	180	0	0.1
Carbobenzoxy-L-isoasparagine.....	4.9	180	1	0.2
"	7.4	180	2	0.4
Carbobenzoxy-L-serinamide‡......	5.2	120	6	0.2
"	7.7	60	2	0.8
		120	5	1.4
Benzoylglycinamide‡.............	5.1	90	1	0.3
		180	2	0.4
"	7.8	90	3	1.2
		180	6	2.2
"	7.7*	180	0	0.05
Carbobenzoxy-L-methioninamide§..	5.5	180	5	0.3
" ..	7.8	180	7	1.3
Carbobenzoxy-D-methioninamide§..	7.5	180	0	0.03
None...........................	7.5	120	0	0.03

* No enzyme present.

† No cysteine present.

‡ 0.3 cc. of methanol added per cc. of test solution to dissolve the substrate. In the presence of 30 per cent methanol, the rate of hydrolysis of synthetic substrates by papain is decreased.

§ This substance was not completely in solution at the start of the experiment even with the addition of 0.3 cc. of methanol per cc. of test solution.

zoylglycinamide, carbobenzoxy-L-serinamide, and carbobenzoxy-L-methioninamide (11). It is of interest that carbobenzoxy-L-isoasparagine,

which has been found to be relatively resistant to the hydrolytic action of papain at pH 5 (11), exhibits only slight transamidation with hydroxylamine in the presence of the enzyme preparation. Also, carbobenzoxy-D-methioninamide was unable to participate in the enzyme-catalyzed replacement reaction. The conclusions as to the specificity of the enzymatic components of papain, drawn from studies of the hydrolysis of synthetic substrates, also apply, therefore, to the catalysis of transamidation reactions by these enzymes. This may be taken as additional evidence for a fundamental similarity, if not identity, in the mechanism of enzymatic catalysis of hydrolysis and replacement, as suggested earlier in this communication and elsewhere (1).

It will be noted from Table II that, with benzoyl-L-argininamide as the substrate, a shift in pH from 6.8 to 7.5 does not increase the extent of transamidation with hydroxylamine to an appreciable degree. This result may be contrasted with the effect of pH on the transamidation with isotopic ammonia. The explanation for this difference in the behavior of these two replacement agents appear to lie in the fact that the pK of the NH_4^+ ion is about 9.4, while that of the NH_3OH^+ ion is about 6. From this it would follow that it is the uncharged NH_2OH molecule which participates in the enzyme-catalyzed transamidation reaction.

Since benzoyl-L-arginine and carbobenzoxy-L-glutamic acid do not show appreciable hydroxamic acid formation under conditions in which the corresponding amides react extensively, the possibility of prior hydrolysis of the amide to the acylamino acid and subsequent synthesis of the hydroxamic acid appears to be ruled out. Also, in the absence of a suitable substrate, the enzyme preparation, after incubation with hydroxylamine, gives only a negligible color with ferric chloride. The fact that substitution of the citrate-phosphate buffer by veronal buffer did not alter the results to a significant extent indicates that the added phosphate is not specifically involved in the transamidation reaction (Table III). A specific rôle of cysteine also is unlikely, since transamidation with hydroxylamine could be demonstrated in experiments in which this activator was replaced by cyanide.

The data in Table III describe the extent of papain-catalyzed transamidation at pH 7.4 to 7.5 as a function of the initial hydroxylamine concentration. As was to be expected, the highest concentration of hydroxylamine employed (0.2 M) led to the most extensive transamidation; it is significant, however, that the presence of as low a concentration as 0.01 M hydroxylamine was sufficient for appreciable hydroxamic acid formation. It will be seen from the data in Table III that, at the lower hydroxylamine concentrations, the hydroxamic acid level attained a maximum and then receded. This may readily be explained by the fact that

the products of transamidation with hydroxylamine are also substrates for papain, as shown in Table IV, where the rates of the hydrolytic action of papain on benzoylglycylhydroxamic acid and on benzoylglycinamide are compared. In this connection, it may be mentioned that a recent report (12) describes the hydrolysis of benzoyl-L-phenylalanylhydroxamic acid by chymotrypsin. In the transamidation experiments, therefore, when the rate of transamidation exceeds the rate of the enzymatic hydrol-

TABLE III

Effect of Hydroxylamine Concentration on Extent of Papain-Catalyzed Hydroxamic Acid Formation

Substrate, benzoyl-L-argininamide, 0.05 M; enzyme concentration, 0.25 mg. of protein N per cc. of test solution; cysteine concentration, 0.025 M; pH 7.4 to 7.5.

Concentration of hydroxylamine	Time	Ammonia liberation	Hydroxamic acid present
M	min.	μM per cc.	μM per cc.
0.2	20	4	2.2
	70	16	5.5
	145	23	8.2
0.1	20	6	2.0
	70	19	5.2
	145	29	8.0
0.05	20	7	1.4
	70	23	4.1
	145	36	4.4
	225	43	3.6
0.05*	20	6	1.3
	70	19	3.8
	145	34	4.6
0.03	20	8	1.2
	70	27	2.8
	145	42	2.1
	225	46	1.3
0.01	20	13	0.7
	70	39	1.0
	145	49	0.2

* 0.02 M veronal buffer used in place of the citrate-phosphate buffer.

ysis of the newly formed hydroxamic acid, the concentration of hydroxamic acid increases steadily, as at the higher hydroxylamine concentrations (0.1 to 0.2 M) of Table III; when the rate of transamidation falls off to the point at which it is less than the rate of the hydrolysis of hydroxamic acid, a decrease in the hydroxamic acid concentration may be expected. The data in Table III show that the rate of enzymatic hydrolysis of the amide increases as the hydroxylamine concentration is decreased.

The hydroxylamine appears, therefore, to be performing a dual rôle in these experiments, since it acts both as an inhibitor of the enzyme and as a partner in the transamidation reaction.

Preliminary experiments with a preparation of beef spleen cathepsin have shown that enzyme-catalyzed hydroxamic acid formation occurs at pH 6.5 to 7, with benzoyl-L-argininamide as the substrate. As in the case of papain, the extent of transamidation in this pH region is much greater than at pH 5, at which cysteine-activated beef spleen cathepsin II acts optimally in the catalysis of the hydrolytic reaction (13). The catalysis of transamidation reactions by crystalline pancreatic trypsin and chymotrypsin is under investigation.

TABLE IV

Hydrolysis of Benzoylglycylhydroxamic Acid and of Benzoylglycinamide by Papain

Concentration of substrates, 0.05 M; enzyme concentration, 0.25 mg. of protein N per cc. of test solution; cysteine concentration, 0.025 M; methanol, 0.3 cc. per cc. of test solution.

Substrate	pH	Time	Hydrolysis
		hrs.	*per cent*
Benzoylglycylhydroxamic acid*	5.9	1	21
		3	33
		5	39
" "	7.3	1	22
		3	37
		5	42
Benzoylglycinamide†	5.8	3	19
		5	31
"	7.8	3	19
		5	32

* Hydrolysis followed by colorimetric estimation of hydroxamic acid.
† Hydrolysis followed by measurement of ammonia liberation.

DISCUSSION

The experiments reported above give support to the view that proteolytic enzymes of plant and animal tissues catalyze transamidation reactions. The possible rôle of such reactions in the biosynthesis of the peptide bonds of proteins and of naturally occurring peptides has been discussed previously (1). A significant result of the present study is the disparity between the pH optima for hydrolysis and for transamidation in the action of several intracellular proteinases. This finding suggests that, at physiological pH values, transamidation may represent a major reaction in living cells; upon death of the cell, and the accompanying shift of pH to more acid values, the action of the intracellular protea-

lytic enzymes may be almost exclusively a hydrolytic one. The further experimental exploration of this working hypothesis should throw valuable light on the rôle of these enzymes in the biosynthesis of peptide bonds.

The view that enzymes usually classified as catalysts of hydrolytic reactions also catalyze replacement reactions appears to have validity for the acid and alkaline phosphatases (14, 15) and for intestinal dipeptidase (16). Also, the enzymatic synthesis of γ-glutamohydroxamic acid and of β-aspartohydroxamic acid, from glutamine and asparagine respectively, has recently been reported by Waelsch et al. (17). In their studies, which bear an immediate relationship to the results described in the present communication, a cell-free extract of Proteus vulgaris was used as the enzyme preparation; no information was given by them (17) as to the hydrolytic activity of the extract toward the two amides. Stumpf and Loomis (18) also have reported the enzymatic synthesis of γ-glutamohydroxamic acid from glutamine and hydroxylamine; in their experiments, pumpkin seedlings served as the source of the enzyme preparation. Furthermore, the studies of Hehre (19), Monod and Torriani (20), Doudoroff et al. (21), and others have demonstrated the biological occurrence of replacement reactions involving glycosidic linkages. It is clear, therefore, that increasing attention is being given to the possible physiological importance of enzyme-catalyzed reactions in which one participant of an amide (or peptide) bond, of an ester linkage, or of a glycosidic bond is replaced by another closely related molecular species, with a relatively small change in free energy (1). In the face of the data presented in this communication, and by other investigators, it will be important to examine the possibility that some of the enzymes now considered to function solely as catalysts in hydrolytic reactions may have an important physiological rôle in the catalysis of replacement reactions.

EXPERIMENTAL

In the conduct of the enzyme experiments, mixtures of 0.05 M citrate and of 0.05 M phosphate were employed, except as otherwise stated. The temperature was maintained at 38.1°. The papain preparation was obtained from crude dried papaya latex by the method of Grassmann (22). The beef spleen cathepsin preparation was obtained by fractional precipitation with ammonium sulfate, as described previously (13).

Measurement of Ammonia Formation—0.1 cc. portions of the incubation mixture were placed in duplicate Conway vessels (A. H. Thomas Company, No. 4427-F), which contained 1 cc. of 2 per cent boric acid reagent (23) in the center well. The ammonia was liberated by mixing the test sample with 1 cc. of saturated potassium carbonate solution.

The vessels were kept at room temperature (20–25°) for 3 hours, and the contents of the center wells were titrated with 0.0033 N sulfuric acid. Control experiments showed that, under these conditions, the acylamino acid amides employed did not undergo deamidation, and that the presence of hydroxylamine in the incubation mixture did not interfere with the accuracy of the ammonia determinations.

Colorimetric Determination of Hydroxamic Acid Concentration—The procedure employed involved a slight modification of the method described by Lipmann and Tuttle (6). 1 cc. of the incubation mixture was added to 1 cc. of 20 per cent trichloroacetic acid; 1 cc. of 5 per cent ferric chloride in 0.1 N hydrochloric acid was then added, and the volume was adjusted to 5 cc. The mixture was centrifuged, and the optical density of the clear supernatant solution was read within 15 minutes of color development in a Klett-Summerson colorimeter with a No. 54 filter. Additional hydroxylamine was not introduced during the development of the color (6, 24), since this was not found to increase the constancy of the readings to a significant extent. Control experiments showed that the presence of the amounts of phosphate, cysteine, or methanol used in these studies affected the color development only slightly (2 to 5 per cent). The procedure described above also was employed in the determination of the calibration curves for the authentic hydroxamic acids.

Benzoylglycylhydroxamic Acid—3 gm. of benzoylglycinamide (25) were heated under a reflux with 30 cc. of methanol which contained 2 gm. of free hydroxylamine (26). After 1 hour, 50 cc. of water were added, and the solution was acidified to about pH 6 with concentrated hydrochloric acid. On chilling the solution, the product (1.7 gm.) crystallized. After recrystallization from water, the substance melted at 158–159°.

$$C_9H_{10}O_3N_2 \ (194.2). \ \text{Calculated, N 14.43; found, N 14.42}$$

Benzoyl-L-alanylhydroxamic Acid—1.4 gm. of benzoyl-L-alanine methyl ester were heated with 1 gm. of hydroxylamine in 10 cc. of methanol as described for the glycyl compound. After the solution was diluted with water, acidified, and chilled, 1.0 gm. of the product was obtained. M.p., 169–170°.

$$C_{10}H_{12}O_3N_2 \ (208.2). \ \text{Calculated, N 13.46; found, N 13.45}$$

Benzoyl-DL-leucylhydroxamic Acid—This substance was prepared from 1.7 gm. of the corresponding ethyl ester in the same manner as the alanyl compound. Yield, 1.5 gm.; m.p., 160–161°.

$$C_{13}H_{18}O_3N_2 \ (250.3). \ \text{Calculated, N 11.20; found, N 11.11}$$

Acethydroxamic Acid—This substance was prepared in the manner described by Miolati (27).

$$C_2H_4O_2N \text{ (75.1)}. \quad \text{Calculated, N 18.66; found, N 18.49}$$

Benzhydroxamic Acid—This substance was prepared in the manner described by Jones and Hurd (28). M.p., 123–124°.

The authors are greatly indebted to Dr. Henry D. Hoberman and to Mr. Joseph Doolittle for their generous cooperation in the conduct of the isotope experiments.

SUMMARY

1. Cysteine-activated papain catalyzes the replacement of the amide N of benzoylglycinamide by N^{15}, introduced into the reaction as isotopic ammonium N. This transamidation is favored by a shift in pH from 5 to 8.

2. Cysteine-activated papain catalyzes the replacement of the amide NH_2 group of a number of acylamino acid amides by the —NHOH group of hydroxylamine. The hydroxamic acids so formed have been estimated colorimetrically. As with the isotope experiments, a shift in pH from 5 to 7.5 markedly increases the extent of transamidation. Similar results have been obtained with a preparation of beef spleen cathepsin.

3. The possible significance of these findings for the study of the biosynthesis of peptide bonds is discussed.

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ANTAGONISTS OF NUCLEIC ACID DERIVATIVES

II. REVERSAL STUDIES WITH SUBSTANCES STRUCTURALLY RELATED TO THYMINE

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One of the major problems of antimetabolite studies is the evaluation of the rôle of "secondary reversing agents" (1). In a system in which there exists a competitive relationship between a metabolite and its antimetabolite, the effect of a secondary reversing agent is to increase the ratio of antimetabolite to metabolite required to produce a given inhibition; the secondary reversing agent cannot, however, replace the metabolite. One of the better known examples is the effect of methionine and purines on the sulfanilamide-p-aminobenzoic acid antagonism (2–5). Kohn and Harris (3) have suggested that methionine acts as a reversing agent in this system because the synthesis of methionine involves p-aminobenzoic acid as a catalyst. This type of explanation has been employed by Shive and associates (5, 6) and applied to the identification of the products of enzyme systems of intact cells.

A variety of activities for *Lactobacillus casei* is found among pyrimidines structurally related to thymine. Several 5-methylpyrimidines have thymine-like activity (7, 8), while a number of uracils, substituted in the 5 position with groups other than alkyl, are inhibitory (7, 8). Among these inhibitors, there exist at least four distinct types which can be differentiated on the basis of reversal experiments.

During the course of these reversal studies, the chemical specificity of secondary reversing action was examined in a number of systems and found to be so highly unspecific as to render this type of action essentially uninterpretable in terms of specific enzymic activities, prosthetic groups, and products (9).

EXPERIMENTAL

The experiments with *L. casei* were carried out as described previously (8). By adding supplements to the basal medium (O), the following media are obtained: OT, O + 1 γ of thymine per ml.; OFA, O + 0.046 mμgm. of folic acid per ml.; P, O + 10 γ of adenine sulfate per ml.; PT, P + 1 γ of thymine per ml.; and PFA, P + 0.046 mμgm. of folic acid per ml. The cultures were incubated 68 hours at 37°.

Results

Compounds which may be viewed as structurally related to thymine through the replacement of the methyl group by atoms or groups other than alkyl generally have been found to have inhibitory effects on *L. casei* (7). These inhibitors appear to fall into four main groupings which may be represented by 5-hydroxy-, 5-amino-, 5-nitro-, and 5-bromouracils. As shown in previous papers (7, 8), 5-hydroxy- and 5-aminouracils inhibit growth in both the thymine- and folic acid-containing media. 5-Nitro-uracil, on the other hand, affects primarily the growth with folic acid, while only slightly inhibiting growth with thymine, and 5-bromouracil greatly depresses growth with thymine but slightly stimulates growth with folic

TABLE I

Effect of Uracil on Inhibitory Activity of 5-Hydroxyuracil

5-Hydroxyuracil	Uracil	Acid production, ml. 0.1 N acid per 10 ml.	
		PT medium	PFA medium
γ *per ml.*	γ *per ml.*		
0	0	5.5	7.7
0	0.02		7.7
0	0.5	5.6	6.8
0	2.5	5.5	6.5
100	0	0.7	0.9
100	0.02	0.9	1.0
100	0.50	4.2	7.2
100	2.5	5.5	7.2

acid (and adenine). This paper is concerned primarily with reversal experiments.

The data for the action of 5-hydroxyuracil on *L. casei* are given in Table I. In the presence of 5-hydroxyuracil, growth is suppressed both in the thymine (PT) and the folic acid (PFA) media, but can be restored by uracil, which has little effect when added alone.

A second type of inhibitor may be represented by 5-aminouracil (Table II) which also inhibits growth both with thymine and with folic acid. These inhibitions are reversible by either metabolite. With thymine the inhibition approximates the competitive type (equal effects at the same aminouracil-thymine ratio). However, with folic acid the analogue-metabolite relationship assumes an exponential form.

The effects of 5-bromouracil seem to be directed chiefly against thymine, since it interferes with the growth of *L. casei* in media with thymine, but has a slight stimulatory effect on growth with folic acid in the presence of

purines (7, 8). The data of reversal experiments involving 5-bromouracil and thymine are given in Table III. It is seen that constant growth effects are obtained, at different concentrations, at the same ratio of bromouracil to thymine; *i.e.*, that the inhibition is competitive over the limited range of concentration which could be investigated.

TABLE II

Effects of Folic Acid and Thymine on Inhibitory Activity of 5-Aminouracil

		5-Aminouracil, γ per ml.				
	Nutrilite	0	1	5	25	125
		Acid production, ml. 0.1 N acid per 10 ml.				
	mμgm. per ml.					
Folic acid (O medium)	0.022	5.2	3.2	1.4	1.1	0.7
	0.112	10.2	7.2	5.0	3.6	2.1
	0.560	13.5	13.0	11.5	10.4	7.9
	2.80	13.7	13.5	13.1	12.7	12.4
	14.4	13.7	13.6	13.6	13.5	13.2
	γ per ml.					
Thymine (P medium)	0.1	4.8	4.1	2.0	0.4	0.4
	0.5	5.3	4.7	3.7	2.6	0.4
	1.0	5.8	5.1	4.8	3.9	2.0
	5.0	6.3	6.2	6.6	6.2	5.1

TABLE III

Effect of Thymine on Inhibitory Activity of 5-Bromouracil

Thymine (P medium)	5-Bromouracil, γ per ml.				
	0	16	40	100	250
	Acid production, ml. 0.1 N acid per 10 ml.				
γ per ml.					
0.16	6.5	4.5	3.2	2.8	2.8
0.40	8.0	6.1	4.2	2.8	2.7
1.0	8.8	8.1	6.5	4.3	3.4
2.5	8.8	9.0	7.8	6.0	3.8

The anti-folic acid effects of 5-nitrouracil are illustrated by the data of Table IV. This pyrimidine depresses growth at all levels of folic acid and the ratio of inhibitor to metabolite which produces a given inhibition is reasonably constant. In the presence of thymine, which has relatively little effect when added to the control media, the inhibitory effects of 5-nitrouracil are greatly diminished. Thus, in the presence of 100 γ of 5-nitrouracil and 0.112 mμgm. of folic acid, the addition of 1 γ of thymine

per ml. results in an increase in titer from 0.8 to 4,5 ml., while at 2.8 mμgm. of folic acid the increase is from 5.6 to 13.1 ml. However, 5-nitrouracil is known to have a very slight inhibitory effect on growth with thy-

TABLE IV

Reversal of 5-Nitrouracil Inhibition by Folic Acid

Medium	5-Nitrouracil	Folic acid, mμgm. per ml.				
		0.0224	0.112	0.560	2.80	14.4
		Acid production, ml . 0.1 N acid per 10 ml.				
	γ *per ml.*					
O	0	4.7	10.8	14.4	14.6	14.6
	16	0.9	2.6	6.5	12.7	13.2
	40	0.6	1.2	4.4	6.0	9.8
	100	0.5	0.8	2.4	5.6	8.0
	250	0.5	0.6	0.7	4.0	6.4
OT	0		9.2		14.0	14.1
	100		4.5		13.1	13.4

TABLE V

Effects of Various Uracil Derivatives on Inhibitory Action of 5-Nitrouracil

Compound, 100 γ per ml.	Absence of 5-nitrouracil		Presence of 5-nitrouracil, 100 γ per ml.	
	OFA	PT	FA (0.112 mμgm. per ml.)	FA (0.56 mμgm. per ml.)
	Acid production, ml. 0.1 N acid per 10 ml.			
Controls.................................	6.0	6.5	0.65	0.7
5-Chlorouracil...........................	5.1	1.6	4.05	10.65
5-Bromouracil............................	4.8	2.5	6.0	13.8
5-Iodouracil.............................	4.6	5.0	3.0	9.65
5-Cyanouracil............................	4.8	7.2	3.8	5.2
4-Methyl-5-bromouracil...................	5.3	1.3	2.7	9.35
5,5-Dichloro-4-hydrohydroxyuracil.........	5.1	2.9	3.85	12.85
5,5-Dibromobarbituric acid...............	6.5	3.8	0.7	0.7
4-Hydroxy-5-methylpyrimidine.............	5.4	6.4	2.6	7.3
			Absence of 5-nitrouracil	
Controls.................................			8.3	14.0

mine, and thymine itself slightly depresses growth with folic acid in the absence of purine (8).

It has been reported that bromouracil, like thymine, is capable of a partial reversal of the nitrouracil inhibition of *L. casei* (9). That this is

not a unique property of bromouracil is indicated by the data of Table V, which show the effect of nine analogues on the nitrouracil inhibition. With the exception of 5,5-dibromobarbituric acid, these compounds by themselves have a slight inhibitory effect on growth in the presence of folic acid (OFA medium).[1] On the other hand, some diversity of effects is shown in the thymine (PT) medium. Here, a number of the compounds, like bromouracil, are inhibitory, but cyanouracil is slightly stimulatory and 4-hydroxy-5-methylpyrimidine is essentially inactive. The last two columns of Table V deal with the effects of these substances on the growth of *L. casei* in the presence of nitrouracil (100 γ per ml.) and folic acid (FA+, 0.112 mμgm., and FA++, 0.56 mμgm. per ml.). It is seen that

TABLE VI

Reversals of Inhibitions by Various Substances

Compound	Concentration	OFA	FA (2.8 mμgm. per ml.)	PFA	OFA + thymine, 1 γ per ml.	OFA + uracil, 2.5 γ per ml.	OFA + 5-bromouracil, 100 γ per ml.
			Acid production, ml. 0.1 N acid per 10 ml.				
	γ per ml.						
Controls.....................		5.2	14.0	7.7	4.8	5.45	3.8
5-Nitrouracil.................	100	0.5	5.6	0.8	2.75	0.45	2.15
5-Hydroxyuracil..............	100	0.5	0.8	0.7	0.5	5.3	2.8
5-Aminouracil................	100	0.55	12.6	3.0	2.6	0.6	2.7
4-Aminofolic acid...........	0.00025	0.50	12.8	6.0	0.6	0.45	1.9
4-Amino-N^{10}-methylfolic acid..	0.0001	1.8	13.3	6.9	2.5	3.8	2.85

no growth results in either medium in the presence of both inhibitor and nutrilite. However, on the addition of bromouracil or, in fact, any of the analogues listed, with the exception of 5,5-dibromobarbituric acid, a considerable restoration of growth occurs.

The ability of 5-bromouracil to restore growth in the presence of folic acid and an inhibitor is not confined to the system containing nitrouracil. This is shown in Table VI in which the effects of four secondary reversing agents, adenine, thymine, uracil, and 5-bromouracil, on five inhibitors are presented. In each instance sufficient inhibitor to abolish growth was added to the medium (OFA). Additional folic acid (FA) causes increased growth in the presence of four of the inhibitors but fails to influence the inhibition caused by 5-hydroxyuracil. The addition of adenine (PFA)

[1] It should be noted that while bromouracil is stimulatory in the medium containing folic acid and purine it is inhibitory when the purine is omitted. In both these respects it resembles thymine.

partially restores growth in the presence of 5-aminouracil or the structural analogues of folic acid. The superimposition of thymine in the OFA medium slightly depresses growth of the control but relieves the inhibitions produced by nitrouracil, aminouracil, and 4-amino-N^{10}-methylfolic acid. On the other hand, uracil is effective in the reversal of the inhibition caused by 5-hydroxyuracil and 4-amino-N^{10}-methylfolic acid, but does not affect the action of nitrouracil or aminouracil. Finally, 5-bromouracil depresses growth with folic acid (in the absence of purine) but produces some restoration of growth in the presence of each of the inhibitors.

DISCUSSION

Reversal experiments demonstrate a number of competitive inhibitions between thymine and certain structural analogues of thymine. Thus thymine with either 5-aminouracil (Table II) or 5-bromouracil (Table III) gives essentially identical growth at the same ratio of metabolite to antimetabolite over the range of concentration which could be studied. The structural similarity of the inhibitors to thymine lends credence to the belief that the analogues are competing with thymine for some essential constituent of the microorganism.

The competition between 5-nitrouracil and folic acid is more difficult to interpret. The products formed by the obvious chemical reactions of nitrouracil (e.g. 5-hydroxyuracil, 5-aminouracil) are known and their biochemical activities have been investigated and found to differ from those of nitrouracil. When it was first discovered that the addition of thymine to the 5-nitrouracil-folic acid system results in a considerable restoration of growth, the following explanation was considered. If it is assumed that folic acid has a catalytic function in the biosynthesis of thymine and purine, as is suggested by the nutritional equivalence of the bases to the vitamin, it might be assumed that the function of folic acid dealing with the synthesis of thymine is blocked by 5-nitrouracil, while that dealing with the synthesis of purines remains operative. The addition of thymine to such a system, therefore, would result in restoration of growth.

This explanation appeared plausible until the specificity of the action of thymine was investigated. It was found that a considerable variety of pyrimidines, such as those listed in Table V, have essentially the same action. Since the majority of these do not otherwise resemble thymine, considerable doubt is cast on the significance of the reversing action of thymine, and on the type of reasoning outlined above as a possible explanation of this action.

The demonstration that 5-bromouracil effects a partial reversal of a variety of inhibitors (Table VI) and does not necessarily resemble thymine in this respect again emphasizes that inferences and deductions drawn from

the action of secondary reversing agents should be regarded as suggestions only and not as definitive conclusions (9).

SUMMARY

Competitive inhibitions between thymine and 5-aminouracil and 5-bromouracil, between folic acid and 5-nitrouracil, and between uracil and 5-hydroxyuracil in the growth of *Lactobacillus casei* are demonstrated.

The activity of thymine in the reversal of the inhibitory effects of nitrouracil is shown to be highly unspecific. Moreover, 5-bromouracil has a secondary reversing action in a number of competitive inhibitions of *L. casei*. In the systems examined, therefore, the activities of secondary reversing agents are not interpretable in terms of specific enzymes and enzymic products.

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ANTAGONISTS OF NUCLEIC ACID DERIVATIVES

III. THE SPECIFICITY OF THE PURINE REQUIREMENT OF LACTOBACILLUS CASEI

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The purine requirement of *Lactobacillus casei* is an obligate requirement when the microorganism is grown with thymine, whereas with folic acid (FA) purines have only a slight stimulatory effect (1). Therefore, by the use of a medium containing thymine in excess of the growth requirement it is possible to test purine derivatives and possible precursors for their ability to satisfy this requirement of the *Lactobacillus*. This type of experiment is a useful preliminary to the search for antagonists (1), since by this means structural configurations of importance to the organism often can be identified. Thus certain functional groups were found to reduce activity by the same proportion when introduced into either thymine-like or inhibitory pyrimidines (2).

The experiments to be presented herein deal with the usual natural purines, position isomers of these, and a number of purines substituted with unnatural groupings.

EXPERIMENTAL

Microbiological—The basal medium previously described (1) was supplemented with 1 γ of thymine per ml. (OT medium (1)). The derivative to be tested was added at 5 and 100 γ per ml. Except as specified, the cultures were incubated 68 hours at 37°.

Compounds—Unless otherwise indicated, the compounds were synthesized in these laboratories, in the main by known methods. The syntheses of a few of these will be reported elsewhere at a later date. Usually the ultraviolet absorption spectra served as adequate criteria of purity. To a considerable extent the microbiological assay procedure serves as a check on the purity of the compounds. For example, contamination with a trace of folic acid activity (presumably arising from a water supply contaminated with molds or diatoms) is immediately apparent in the O, PO, and PT media described earlier (1). In the few instances in which such contamination was observed it could be removed by the recrystallization of the substance from water with charcoal in the pH range of 3 to 5. In several instances the paper chromatographic method of Hotchkiss (3) was used to determine the homogeneity of the substance in question.

TABLE I

Purines in Growth of Lactobacillus casei

Compound	Concentration, mg. per 10 ml.	Acid production, ml. 0.1 N acid per 10 ml.	Compound	Concentration, mg. per 10 ml.	Acid production, ml. 0.1 N acid per 10 ml.
Adenine sulfate	0.1	5.5	2,8-Dihydroxypurine†	1.0	0.4
2-Methyladenine*	1.0	0.8	8-Methylxanthine	1.0	0.5
7-Methyladenine	1.0	0.6	6,8-Dihydroxypurine	1.0	0.8
2,7-Dimethyladenine*	1.0	0.8	Adenosine	0.5	4.8
2,9-Dimethyladenine*	1.0	0.8	Adenylic acid	0.5	3.1
2-Aminopurine†	1.0	0.6	Guanosine	0.5	3.4
6-Methylaminopurine	0.8	5.2	Guanylic acid	0.5	6.9
Guanine hydrochloride	0.1	5.3	Inosine triphosphate	1.0	3.0
1-Methylguanine	1.0	4.0	Uric acid	1.0	0.6
7-Methylguanine	1.0	0.4	2-Amino-6,8-dihydroxy-purine†	1.0	0.5
8-Methylguanine	1.0	1.6			
1,7-Dimethylguanine	1.0	0.4	2,6-Diamino-8-hydroxy-purine	1.0	0.6
2-Hydroxy-6-aminopurine	1.0	0.3			
			6-Amino-2,8-dihydroxy-purine†	1.0	0.5
2-Amino-8-hydroxypurine	1.0	0.4			
			2-Amino-6-hydroxy-8-thiolpurine†	1.0	0.9
6-Amino-8-hydroxypurine	1.0	0.5			
			2-Thiol-6-hydroxy-purine	1.0	2.7
2,6-Diaminopurine†	1.0	0.4			
Hypoxanthine	0.1	5.2	2-Thiol-8-hydroxy-purine†	1.0	0.5
2-Methylhypoxanthine*	1.0	0.5			
7-Methylhypoxanthine	1.0	0.6	2-Thiol-6-aminopurine†	1.0	0.3
2,9-Dimethylhypoxanthine*	1.0	0.6	2-Chloro-6,8-dihydroxy-purine	1.0	0.6
2-Hydroxypurine	1.0	0.6			
8-Hydroxypurine	1.0	0.6	2,6-Dichloro-8-hydroxy-purine	1.0	0.5
Xanthine	0.1	5.3	2,6,8-Trichloropurine pentahydrate	1.0	0.6
1-Methylxanthine	1.0	5.1			
3-Methylxanthine	1.0	0.5	2-Chloro-6-hydroxy-7-methylpurine	1.0	0.5
7-Methylxanthine	1.0	0.7			
1,3-Dimethylxanthine	1.0	0.8	2,8-Dichloro-6-hydroxy-purine	1.0	0.5
1,7-Dimethylxanthine	1.0	0.7			
3,7-Dimethylxanthine	1.0	0.8	2,8-Dichloro-6-amino-purine†	1.0	0.4
1,3,7-Trimethylxanthine	1.0	0.8			
			Control		0.5

* Supplied by Professor A. R. Todd.

† Inhibitor of growth with folic acid (OFA medium (1)).

RESULTS AND DISCUSSION

The results of tests of 52 substituted purines for their ability to supply the purine requirement of *L. casei* are shown in Table I. The natural

purine bases, adenine, hypoxanthine, guanine, and xanthine, are about equally effective. Presumably this indicates the presence of a biochemical mechanism for the interconversion of adenine and guanine and for the amination of the hydroxypurines. Uric acid, on the other hand, is completely inactive.

Nucleosides and nucleotides, such as adenosine, adenylic acid, guanosine and guanylic acid, and inosine triphosphate, on a molar basis are certainly no more and are perhaps less active than the free bases and appear to be used only after degradation.

Methylation of the ring nitrogen atoms can be seen to eliminate activity with the exception of the 1-methyl derivatives, 1-methylguanine and 1-

TABLE II

Pyrimidines As Substitutes for Purines in Growth of Lactobacillus casei

Concentration of purines, 1.0 mg. per 10 ml.

Compound No.	Group at position			Acid production, ml. 0.1 N acid per 10 ml.
	2	5	6	
1	NH$_2$	NH$_2$	OH	0.5
2	"	NHCHO	"	1.1
3	OH	NH$_2$	"	0.8
4	"	NHCHO	"	0.9
5	H	NH$_2$	"	0.6
6	"	NHCHO	"	1.3
7	"	NH$_2$	NH$_2$	0.5
8	"	NHCHO	"	1.9
Control				0.5

methylxanthine. The ability of 1-methylpurines to simulate the unsubstituted derivatives appears to be a quite general phenomenon (4). The activity of these substances as substrates for isolated enzymes (5) indicates that demethylation is not necessarily a prerequisite for biological activity. Perhaps it should not be assumed, without proof, that these substances may not be incorporated into the nucleic acid molecule.

In the xanthine group, data are presented for an almost complete series of nuclear *N*-methyl derivatives. The general pattern of inactivity, in all save the 1-methyl derivative, seen in this series finds no exceptions in the derivatives of the other bases such as 7-methylguanine, 1,7-dimethylguanine, 7-methyladenine, and 7-methylhypoxanthine.

The introduction of a methyl group by way of a C—C bond in position 2 appears to eliminate the activity, as in 2-methyladenine and 2-methylhypoxanthine. However, 8-methylguanine has limited guanine-like activity, although 8-methylxanthine is inactive.

In contrast to the nuclear methylated adenines, 6-methylaminopurine has about 10 per cent of the activity of adenine.

The high degree of specificity of the natural purines is indicated by reference to a number of position isomers. Thus 2-aminopurine, an isomer of adenine, has only inhibitory effects (6), while 2-hydroxy- and 8-hydroxypurine, isomers of hypoxanthine, are inactive. The guanine isomers, 2-hydroxy-6-aminopurine (isoguanine), 2-amino-8-hydroxypurine, and 6-amino-8-hydroxypurine, also have no activity. 6,8-Dihydroxypurine is inactive but 2,8-dihydroxypurine, another xanthine isomer, is inhibitory.

No substances capable of supporting growth were found among the functional derivatives of uric acid; however, several inhibitory substances, such as 2-amino-6,8-dihydroxypurine (8-hydroxyguanine) and 6-amino-2,8-dihydroxypurine (8-hydroxyisoguanine), were found.

Among the thiolpurines, several were inhibitory, although 2-thioxanthine was capable of supporting growth at high concentrations.

Chloropurines, in general, were found to be essentially inactive, except for 2,8-dichloroadenine, which showed inhibitory action.

Eight pyrimidines, which are intermediates in Traube's synthesis of the four natural purines (7–9) were tested for their ability to replace purines (Table II). In the final steps of the chemical synthesis a 4,5-diaminopyrimidine is formylated in position 5 and the 4-amino-5-formamidopyrimidine is then converted to the purine by closure of the imidazole ring.

X, Y = H, OH, NH₂.

The results given in Table II appear to show that the precursors of guanine (Compound 2), hypoxanthine (Compound 6), and adenine (Compound 8) possess some purine-like activity. However, the activities are of a low order and could result from the presence of less than 1 per cent of the corresponding purine. A somewhat greater activity for the guanine precursor was reported earlier (1). Further purification of this substance, meanwhile, has resulted in a considerable diminution in its apparent activity. From this, and similar experiences with the adenine and hypoxan-

thine precursors, it seems probable that the apparent activities of the formamidopyrimidines may be attributed to impurities.

We are indebted to Henry VanderWerff for the microbiological assays, and to Professor A. R. Todd for a number of the compounds.

SUMMARY

The specificity of the purine requirement of *Lactobacillus casei* grown in a medium containing thymine has been examined. Position isomers of the natural purines do not support growth but often act as inhibitors. Among the methylated purines which were studied, only 1-methylguanine, 1-methylxanthine, 8-methylguanine, and 6-methylaminopurine show some degree of purine-like activity.

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THE FORMATION OF GLYCOGEN FROM C¹⁴-LABELED GLYCINE*

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It was demonstrated long ago (1) that glycine gives rise to increased glucose excretion in the phlorhizinized dog. There was some dispute concerning its ability to form liver glycogen until it was found that this occurs at a relatively slow rate in comparison with glucose and other substances, reaching significant levels only after a period of hours (2). Various investigators (3, 4) have suggested, for a number of reasons, that a direct conversion of the carbon of glycine to glucose does not take place and that a shift in the metabolic equilibrium liberated other glycogenic substances. Using isotopic (C¹³) glycine labeled in the carboxyl carbon, Olsen *et al.* (5) concluded from the isotope content of the liver glycogen that glycine may enter into the formation of glycogen in a small degree but primarily promotes the formation of hepatic glycogen from other body constituents. Sakami (6) has also shown that glycine carbon may participate in glycogen formation but gives no evidence as to what extent. The question of the relative importance of the α and carboxyl carbons of glycine in forming glycogen remains unanswered.

EXPERIMENTAL

Synthesis of Radioactive Glycine—The glycine labeled with C¹⁴ in the carboxyl carbon atom and glycine labeled in the α-carbon atom were prepared by the method of Ostwald (7) from the correspondingly labeled acetic acids (8, 9). The carboxyl and α-carbon-labeled glycines had a specific activity of 2100 and 2300 c.p.m. per mg. of total glycine carbon respectively, measured as $BaC^{14}O_3$.

Degradation of Glucose—The glycogen (as glucose) was subjected to Fleischmann's bakers' yeast fermentation to give ethyl alcohol and carbon dioxide (carbon atoms 3 and 4). The ethyl alcohol was oxidized to acetic acid and further degraded by pyrolysis of the barium salt (10).

Measurement of Radioactivity—The radioactivity measurement of the glycine and glycogen samples was made on $BaC^{14}O_3$ obtained from dry combustion. In most cases the samples were of infinite thickness except when insufficient amounts of material were available. In these instances self-absorption curves were used for correcting to infinite thickness (11, 12).

Biological Experiments—The animals, 100 to 150 gm. male albino rats raised on Purina laboratory chow, were fasted 24 hours before administration of the radioglycine by stomach tube. The animals were immediately placed in a metabolism chamber similar to that described by Armstrong *et al.* (13). The expired CO_2 samples were collected and precipitated as

TABLE I

General Experimental Data

Rat No.	Weight after fast	Substance administered	Amount administered*	Liver weight	Liver glycogen† (as glucose)
	gm.		*mg.*	*gm.*	*mg.*
1‡	100	Carboxyl carbon-labeled glycine	530	4.2	122
2	92	" " "	520	3.6	41
3	100	α-Carbon-labeled glycine	530	3.6	33
4	144	" "	700	5.3	70
5	96	Glycine + $NaHC^{14}O_3$	530	3.7	58

* Substance administered on basis of 1 cc. of 3 M glycine solution per sq. dm. body surface.

† 24 hour fasted control animals deposited on the average of 1 to 2 mg. of glycogen.

‡ Fasted 48 hours instead of 24.

$BaC^{14}O_3$ over 2 hour periods except for the eighth period which was 3 hours. At the end of 17 hours the animal was removed from the chamber and sacrificed for analyses. Although each animal was divided into three fractions (liver, the remaining visceral organs, and carcass) for fatty acid and glycogen determinations, this report is concerned only with the liver glycogen. Glycogen was isolated from liver dissolved in 30 per cent KOH according to the method of Good, Kramer, and Somogyi (14).

In addition to the usual 24 hour fasted control runs in which the liver glycogens were determined, the extent of CO_2 fixation was studied by giving an animal inert glycine and radioactive $NaHC^{14}O_3$. The $NaHC^{14}O_3$ was administered by stomach tube at the beginning of each 2 hour period, the total counts at each injection being comparable to that excreted in the same period by the animals to which carboxyl glycine was administered. The experimental data are shown in Tables I, II, and III.

DISCUSSION

The radioactivity data which are recorded in Tables II and III show highly significant differences in the C^{14} content of liver glycogen and CO_2 excretion obtained by feeding carboxyl and α-carbon-labeled glycine. The specific activity of the total glycogen carbon obtained by feeding carboxyl-

TABLE II

Distribution of Radioactivity in Liver Glycogen

Rat No.	Substance administered	Per cent of administered counts recovered in glycogen	Specific activities*			
			Glycogen (glucose)	Carbon atoms of glucose		
				3,4	2,5	1,6
1	Carboxyl carbon-labeled glycine	1.5	123	430	0	0
2	" " "	0.9	180			
3	α-Carbon-labeled glycine	2.2	630			
4	" "	3.0	445	160	590	450
5	Glycine + $NaHC^{14}O_3$	0.4	75	210	0	0

* Counts per minute per mg. of carbon.

TABLE III

Radioactivity Data on Respiratory CO_2

Substance administered	Rat No.	Per cent of administered counts recovered in expired CO_2 in 2 hr. periods*								Total per cent recovered
		1,2	3,4	5,6	7,8	9,10	11,12	13,14	15–17	
Carboxyl carbon-labeled glycine	1	5.6	9.7	8.9	11.7	6.1	3.9	3.7	2.5	52
" "	2	6.6	13.1	16.4	11.5	5.3	3.2	2.3	2.0	60
α-Carbon-labeled glycine	3	0.8	2.5	3.7	6.2	5.2	2.3	2.5	2.1	25
" "	4	1.2	5.0	6.4	11.5	6.9	3.4	2.0	3.2	40
Glycine + $NaHC^{14}O_3$†	5	8.0	8.8	15.0	24.2	13.1	8.2	4.3	4.4	86

* 2 hours each except Period 15–17 which was 3 hours long.

† The radioactive bicarbonate was administered by stomach tube in the beginning of each period. The counts given were comparable to the number expired in the CO_2 in Experiment 1.

labeled glycine shows that the carboxyl carbon of fed glycine supplied approximately 1 carbon for each 28 carbon atoms incorporated into glycogen ($2 \times 2100/151 = 28$; 151 average value). This value is in remarkably close agreement with the findings of Olsen et al. (5) who reported that 1 carboxyl carbon from glycine occurs for every 29 normal carbon atoms of glucose. It is also noted that the specific activity of the glycogen carbon resulting from the administration of the radiobicarbonate and inert

glycine is approximately one-half that obtained by feeding carboxyl-labeled glycine.   Degradation of the glucose (glycogen) obtained from both experiments showed that the labeled carbons were only in the 3rd and 4th positions of the molecule.   Although the control experiments for CO_2 fixation can only approximate the conditions in the animal organism, it is apparent that a substantial number of the carbon atoms of the glycogen arising from the labeled carboxyl carbon of glycine were incorporated by CO_2 fixation.

Examination of the specific activity of the glycogen carbon obtained after feeding α-carbon-labeled glycine shows that 1 carbon atom in every 8.5 carbon atoms in the glycogen molecule was derived from the α-carbon of the fed glycine ($2 \times 2300/538 = 8.5$).   Degradation experiments gave low specific activity values for carbon atoms 3 and 4 as compared to the high values obtained for atoms 1, 6 and 2, 5.   This shows that a considerable fraction of the glycogen deposited after feeding glycine represents glycine carbon included in glycogen by some means other than CO_2 fixation. Although the α-carbon of glycine participates in glycogen formation to a greater extent than the carboxyl carbon of glycine, most of the carbon in glycogen formed as the result of glycine feeding comes from metabolites other than the fed glycine.   When uniformly labeled glucose is administered to rats under conditions similar to these glycine experiments, it was found[1] from specific activity determinations that the isotopic carbon atoms in the liver glycogen obtained 5 hours after the glucose feeding was diluted with 3 to 4 normal carbon atoms in contrast to the 8-fold dilution effect observed by feeding α-labeled glycine.   Both the degree of conversion of the carbon of the fed compound and glycogenesis from the metabolic pool are much greater with glucose than glycine.

Table III shows that the C^{14} is eliminated at a more rapid rate and to a greater extent in the expired CO_2 in the case of the carboxyl-labeled glycine, compared to that after feeding α-carbon-labeled glycine.   It is interesting that the carboxyl carbon is oxidized more rapidly, for there is evidence (15, 16) that the α-carbon of glycine is used to a greater extent for other metabolic functions.

The observation reported here that the α-carbon of glycine enters into glycogen to a greater extent than the carboxyl carbon and the distribution of the C^{14} in the glucose (glycogen) obtained after feeding α-carbon-labeled glycine are compatible with the pathway for the conversion of glycine to glycogen by way of serine which has been shown to be possible by Sakami (6, 17).   In this scheme the α- and β-carbon atoms of serine are both derived from the α-carbon of the glycine.

No evidence is obtained from these experiments relative to the direct

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[1] Wick, A. N., to be published.

conversion of glycine carbon to glycogen carbon *versus* the stimulation of glycogenesis from other sources. Nor is the delay in the deposition of glycogen after glycine feeding (2) explained.

SUMMARY

With the use of the intact rat the specific activity of the glycogen formed after feeding carboxyl-labeled glycine shows that the carboxyl carbon of the administered glycine supplied approximately 1 carbon for each 28 carbon atoms incorporated into glycogen.

Specific activity of the carbon glycogen obtained after feeding α-carbon-labeled glycine shows that 1 carbon atom in every 8.5 carbon atoms in the glycogen molecule was derived from the α-carbon of the fed glycine.

The C^{14} is eliminated at a more rapid rate and to a greater extent in the expired CO_2 in the case of the carboxyl-labeled glycine compared to that obtained after feeding α-carbon-labeled glycine.

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FRACTIONATION OF HUMAN IMMUNE γ-GLOBULIN

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Equine and bovine serum proteins have recently been fractionated by means of a physical method utilizing an electrophoretic adaptation of the principles of the Clusius column (1–4), first described and tested by Kirkwood (5) and Nielsen (6). The method of electrophoresis-convection has now been applied to the fractionation of human γ-globulin. The γ-globulin was prepared by ethanol fractionation (7) from the plasma of individuals hyperimmunized to *Hemophilus pertussis* organisms. The resulting fractions of γ-globulin have been characterized electrophoretically, and the protective antibody activity and agglutinin titer have been measured.

The material used in this investigation was Fraction II of pooled immune human plasma prepared by the Cutter Laboratories from hyperimmunized donors. The apparatus and experimental procedures used in the fractionations reported here were essentially the same as those described for the fractionation of bovine γ-globulin (3, 4). Fractionations were carried out in phosphate buffer, ionic strength 0.1, at a field strength of 1.7 to 2.0 volt per cm. for 48 to 71 hours. The initial protein concentration was 2.0 to 2.8 gm. of protein per 100 ml. Fractions possessing different mean mobilities and isoelectric points were obtained by proper choice of the operating pH.

The agglutinin titers of the fractions were obtained by the Flosdorf method (8) with two different antigen strains. Protective activities of the fractions were determined by mouse protection tests. In these tests, the determinations of the virulence of the challenge and the protective activity of the fractions were carried out simultaneously in order to rule out any change in virulence of the culture. Each of the fractions was injected subcutaneously at doses of 10, 2, and 0.4 mg., with fifteen mice at each level. 6 hours following immunization, a challenge dose of virulent *H. pertussis* organisms was administered to each mouse by intracerebral injection. The virulence of the challenge was determined on forty-eight

mice, twelve mice being tested for each of four dilutions. Death and survivals were recorded after 10 days, those mice substantially paralyzed on the 10th day being considered as dead. The results were analyzed by the method of Reed and Muench (9).

A sample of γ-globulin obtained from the plasma of donors hyperimmunized to *H. pertussis* organisms was carried through three stages of fractionation. The scheme used in this fractionation may be briefly described as follows. In the first stage the γ-globulin was fractionated at pH 8.10. The fractions withdrawn from the top and bottom reservoirs of

TABLE I

Fractionation of Fraction II, Sample 1

Fraction	Per cent of original γ-globulin	Mean mobility,* $-10^5 \times \bar{u}$	Mean isoelectric point†	$\dfrac{-10^5 \times \Delta\bar{u}}{\Delta pH}$	Protective activity,‡ ID_{25} per mg. N	Agglutinin titer§ against	
						Strain P26A	Strain P39A
		$\dfrac{cm.^2\ sec.^{-1}}{volt^{-1}}$	pH	$\dfrac{cm.^2\ sec.^{-1}}{volt^{-1}\ (pH\ unit)^{-1}}$			
Unfractionated γ-globulin..............		1.27	7.55	0.78	1.4 (1.8)	1:1280	1:1280
Top 1................	24	0.88	7.86	1.17	5.0 (2.7)	1:1280	1:1280
" 2................	27	1.23	7.47	1.00	1.3	1:640	1:1280
" 3................	17	1.60	6.96	0.55	2.1	1:640	1:640.
Bottom 3.............	32	1.44	7.28	1.32	1.9	1:640	1:640

* The mean mobilities were determined in barbital buffer, pH 8.6, ionic strength 0.1, and protein concentration 1 per cent.

† The mean isoelectric points were determined in cacodylate-chloride buffer, ionic strength 0.1, and protein concentration 0.5 per cent.

‡ The challenge dose was 100,000 organisms or 250 LD_{50}. The values in parentheses are results of check assays with 100,000 organisms or 910 LD_{50}.

§ The titers are expressed as the titers of 2 per cent solutions of the fractions.

the electrophoresis-convection apparatus are designated as Top 1 and Bottom 1, respectively. Bottom 1 was then refractionated in the second stage at pH 7.78, the resulting fractions being designated as Top 2 and Bottom 2. In the last stage of fractionation, carried out at pH 6.50, Bottom 2 was separated into two fractions, Top 3 and Bottom 3. The electrophoretic and immunological data obtained on the resulting fractions are presented in Table I, where $\bar{u}$ is the mean mobility at pH 8.6 and $\Delta\bar{u}/\Delta pH$ the slope of the mobility-pH curve in the neighborhood of the mean isoelectric point. This fractionation yielded four electrophoretically unique fractions of γ-globulin. These fractions possess a mean mobility spectrum ranging from -0.88×10^{-5} to -1.60×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} at pH 8.6 in barbital buffer and a mean isoelectric point spectrum from 7.86 to 6.96 in cacodylate-chloride buffer.

The results of the assays for protective antibody show that, although all of the fractions possess protective antibody, more protective activity is associated with the fraction of lowest mobility, Top 1, than with the other fractions and the unfractionated γ-globulin. The conclusion that Top 1 possesses more protective activity than the unfractionated material

TABLE II

Fractionation of Fraction II, Sample 2; Electrophoretic and Immunological Properties of Fractions

Fraction	Per cent of Top 1	Mean mobility,* $-10^5 \times \bar{u}$	Mean isoelectric point†	$10^5 \times \beta$‡	Protective activity,§ ID_{50} per mg. N
		$cm.^2 sec.^{-1} volt^{-1}$	pH	$cm.^2 sec.^{-1} volt^{-1}$	
Unfractionated γ-globulin...		1.26	6.90	0.56	1.3
Top 1......................		0.96	7.15		2.1
Bottom 1....................		1.37			
Top 4......................	10	1.28			<0.9
" 5......................	21	1.02	7.28		1.6
" 6......................	23	0.96	7.36	0.53	2.0
" 7......................	17	0.87			2.8
Bottom 7....................	29	0.91	7.46		2.1

* Mean mobilities were determined in barbital buffer, pH 8.7, ionic strength 0.1, and protein concentration 1 per cent.

† The mean isoelectric points were determined in tris(hydroxymethyl)amino-methane buffers, ionic strength 0.1 and protein concentration 0.5 per cent. These buffers were 0.02 N in the hydrochloride of the amine and 0.08 N in sodium chloride. Discrepancies of the order of 0.6 pH unit between isoelectric points determined in cacodylate and tris(hydroxymethyl)aminomethane buffers have been found. These discrepancies are at least in part due to the different temperature coefficients of the pH of these buffers. pH values were measured at 25°, while the isoelectric points were determined at 2°.

‡ The standard deviations of the mobility distributions were determined from reversible boundary spreading experiments. These experiments were carried out in tris(hydroxymethyl)aminomethane buffer at the mean isoelectric points of the proteins.

§ The challenge dose was 100,000 organisms or 690 LD_{50}.

is supported by the results of two independent assays for protective antibody. All four fractions gave nearly the same agglutinin titer against two different antigen strains.

In order to accomplish still further enrichment of protective antibody, a five stage fractionation of a different sample of γ-globulin from hyperimmunized donors was carried out. The results of the electrophoretic and immunological characterization of the various fractions are presented in Table II, where $\bar{u}$ is the mean mobility at pH 8.7 and β the standard

deviation of the mobility distribution in the neighborhood of the mean isoelectric point.

The first stage of fractionation was carried out under the same experimental conditions as were employed in the initial stage of the first fractionation. A composite of the top fractions obtained in ten separate experiments was made. Electrophoretically, this composite resembles Top 1 obtained in the first fractionation as closely as can be expected, considering possible differences in the two samples of unfractionated γ-globulin.[1] Accordingly, this composite has also been designated as Top 1. The immunological assay of the composite Top 1 confirms the previous finding that more protective activity is associated with the low mobility fraction of γ-globulin than with the unfractionated material.

Subsequent stages of fractionation were designed to separate Top 1 into subfractions. The composite Top 1 was fractionated at pH 6.38, the resulting fractions being designated as Top 4 and Bottom 4. Bottom 4 was then fractionated at pH 7.10 to yield Top 5 and Bottom 5. Bottom 5 in turn was fractionated at pH 7.63 to yield Top 6 and Bottom 6. Finally, Bottom 6 was separated into two fractions, Top 7 and Bottom 7, by fractionation at pH 8.00. The mobilities of the resulting subfractions range from -0.87×10^{-5} to 1.28×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} at pH 8.7 in barbital buffer.[2] In agreement with the results of the first fractionation, the immunological characterization of these subfractions revealed that greater protective activity is associated with low mobility fractions than with high mobility fractions.

The rather broad distribution of protective antibody among the fractions may in part be due to the fact that the γ-globulin was prepared from pooled plasma. Of course, there is some question as to whether the protective antibody, if derived from a single donor, might not also exhibit a broad distribution. Several factors might give rise to such a heterogeneity. The first of these is the fact that *H. pertussis* immunity is probably given by several different strains of bacteria. Also, it is conceivable that both bacterial- and toxin-produced antibodies are responsible for immunity.

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[1] The fact that the two samples of γ-globulin possessed the same mean mobility at pH 8.6 does not of course imply that they possessed the same distribution of mobilities.

[2] It is of interest that little or no apparent separation was accomplished in the last two stages of fractionation, despite the fact that the operating pH of successive stages differed by at least 0.4 pH unit. This is somewhat difficult to understand, especially in view of the large amount of transport obtained in these runs and the fact that the standard deviation of the mobility distribution of Top 6 was found to be the same as that of the unfractionated γ-globulin.

SUMMARY
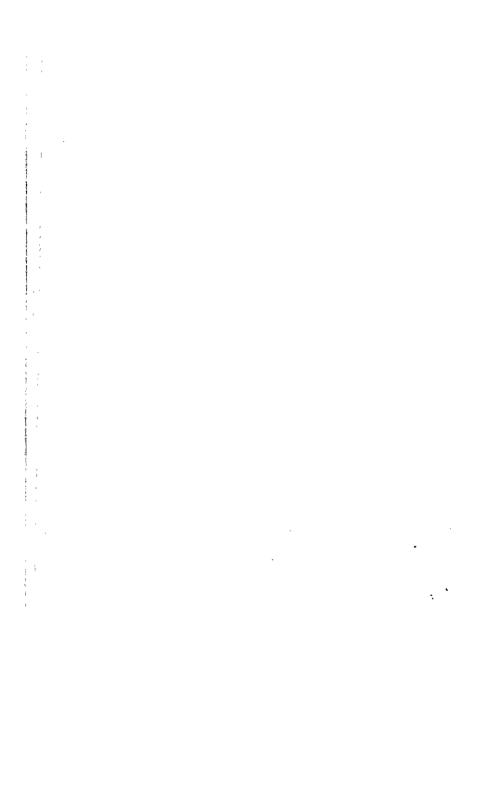
A sample of human antipertussis γ-globulin has been separated into four electrophoretically unique fractions by electrophoresis-convection. These fractions possess a mean mobility spectrum ranging from -0.88×10^{-5} to -1.60×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} at pH 8.6 in barbital buffer and a mean isoelectric point spectrum from 7.86 to 6.96 in cacodylate-chloride buffer. All of the fractions gave nearly the same agglutinin titer against two different antigen strains. Although all fractions showed protective activity, the greatest activity was associated with the fraction of lowest mobility. This was confirmed by the fractionation of a second sample of immune γ-globulin and subsequent subfractionation of the low mobility material.

Addendum—Since preparation of this manuscript, Hink and Johnson (10) have reported their studies on the distribution of antibodies among plasma fractions prepared by ethanol fractionation (7) from the plasma of individuals hyperimmunized to *H. pertussis* organisms. Their results indicate that the protective activity in antipertussis hyperimmunized human plasma occurs almost entirely in the purified γ_2-globulin, Fraction II. Less than half of the *H. pertussis* agglutinins was recovered in Fraction II, which contained 90 per cent of the γ_2-globulin of the plasma. The remaining agglutinin activity was found in Fraction III-1. Their Fraction II was the material used in this investigation.

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ARGINASE, ADENOSINEPYROPHÖSPHATASE, AND RHODANESE LEVELS IN THE LIVER OF RATS*

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Previous studies from this laboratory (2, 3) have dealt with the chemical composition of resting and regenerating rat liver under various dietetic conditions. The major emphasis was placed upon changes in the amount of protein, since the quantity of this tissue component appeared to offer the most appropriate estimate of the functionally effective liver mass. It was felt that supplementation of these data by enzymatic assays could provide more direct information on the functional capability of this organ. Recent reports from several laboratories (4–6) indicate that the change in the activity of an individual enzyme may differ considerably in its magnitude from the change in total liver protein.

The enzyme assays to be reported were carried out on liver tissue from rats which were kept either on a protein-rich, semisynthetic diet or on an analogous protein-free régime for various intervals of time. The animals were then subjected to a partial hepatectomy and sacrificed during the first 8 postoperative days. The present paper deals with the effect of protein starvation on the enzymatic activity of the resting liver tissue that was obtained at operation.

The three enzymes under investigation were selected for metabolic reasons as well as for the sake of accuracy and simplicity of assay. The liver arginase plays a major rôle in the protein catabolism of the animal. The amount and concentration of this enzyme are known to depend on nutritional conditions (4, 5, 7). The enzymatic hydrolysis of adenosine triphosphate is a ubiquitous property of cells which is possibly of importance for the intracellular transfer of energy. Since it is known that liver extracts and homogenates split off both labile phosphorus groups of the nucleotide (8, 9), we shall refer to the enzyme or enzymes involved as adenosinepyrophosphatase rather than as adenosinetriphosphatase. Rhodanese (10) represents a characteristic liver enzyme of about as high an

activity as arginase. No other rat tissue, with the exception of kidney cortex, displays more than 6 per cent of the rhodanese activity of liver tissue (11). The transfer of sulfur from thiosulfate to hydrocyanic acid, which is catalyzed by the enzyme, is not known to be linked to major metabolic processes. Hence rhodanese furnished a useful control system in the study of dietary effects on the levels of liver enzymes.

<center>EXPERIMENTAL</center>

Young male rats of the Wistar strain, weighing approximately 250 gm., were used for the experimental series. They were fed *ad libitum*. The composition of the diets, the technique of the partial hepatectomy, and the handling of the excised liver tissue have been previously discussed by Gurd, Vars, and Ravdin (3).

The major portion of the excised liver tissue was placed for 10 minutes in an ice-cooled container. It was then rapidly minced with scissors and weighed in a glass homogenizer of the Potter-Elvehjem type (12). 4 volumes of ice-cold glass-distilled water were added per gm. of mince and the mixture homogenized for 3 minutes, the tube being immersed in ice water. Upon aliquots of this 20 per cent homogenate, determinations of the total nitrogen, non-protein nitrogen, and total lipides were done as previously described (3).

Arginase was determined according to the directions of Van Slyke and Archibald (13), except that the entire procedure was carried out in War-burg vessels, the urea being determined with the method of Krebs and Henseleit (14). Immediately following the preparation of the 20 per cent homogenate an aliquot was diluted 100-fold with a mixture of equal parts of 0.1 M $MnSO_4$ solution and 0.9 per cent NaCl solution. 1 ml. samples of this 0.2 per cent homogenate were incubated at 56° in rubber-stoppered conical Warburg vessels for a period of 20 minutes. Thereafter the vessels were incubated at 25° with 0.5 ml. of a 5 per cent arginine solution (pH 9.5) for 5 minutes. The arginase action was stopped by the addition of 1 ml. of 0.25 M H_2SO_4. 0.3 ml. of a 5 per cent solution of urease (Arlco) in 0.3 M acetate buffer at pH 5 was placed in the side bulb and 0.5 ml. of 1.5 M sodium acetate solution added to the contents of the main compartment. The urea was then determined manometrically[1] in the usual fashion at 37°. The assays were run in duplicate, which usually agreed within 3 per cent. Whenever deviations greater than 5 per cent were en-

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[1] One vessel, to which the arginine had been added after the sulfuric acid, served as a control. The "blank" pressure, on addition of urease, amounted to but 3 to 4 mm. and did not vary significantly among the individual homogenates. Hence a single thermobarometer control suffices for a set of determinations upon individual homogenates.

countered, the determinations were repeated on one of the following days. This was feasible as the 0.2 per cent homogenates kept their arginase activity for at least 72 hours when stored in the cold.

Seifter *et al.* (5) reported that the arginase activity of liver homogenates greatly depended upon the length of time between excision of the liver and homogenization. We have found no differences in the activity of samples from the same liver specimen when the storage time on ice prior to homogenization was increased from the usual 10 minutes up to 60 minutes.

The incubation of the 0.2 per cent homogenates at 56° for a period of 20 minutes resulted in an increase of but 15 to 25 per cent in the arginase activity. This is considerably less than the increments obtained by Van Slyke and Archibald (15) and Hunter and Downs (16) with dog liver extracts or by ourselves[2] with extracts from acetone-dried rabbit liver. Apparently the arginase activity of rat liver homogenates remained well preserved in the cold manganese saline medium. Roberts (17) reported that for the complete activation of mouse liver homogenate incubation times of 5 hours at 50° were needed. We have found that the arginase activity of homogenates from livers of both protein-fed and protein-depleted rats was not further increased when the incubation period at 56° was extended up to 2 hours. Likewise, Roberts' technique of incubating 1 per cent homogenates at 50° for extended periods of time failed to result in higher activities than those obtained with our standard procedure. It thus appears justifiable to assume that by our method the full arginase activity in resting and regenerating liver tissue, under various nutritional conditions, is determined.

Adenosinepyrophosphatase (Apyrase) was assayed by means of a slight modification of the method of DuBois and Potter (18). 0.2 ml. of a 1 per cent liver homogenate, prepared immediately before use from the 20 per cent homogenate by dilution with redistilled water, was mixed in a micro ignition tube with 0.2 ml. of 0.05 M barbiturate buffer (pH 7.4), 0.2 ml. of 0.012 M adenosine triphosphate solution (ATP), and 0.2 ml. of water or salt solution. The mixture was repeatedly stirred with a small glass rod. After 15 minutes incubation at 37° the reaction was stopped by the addition of 0.2 ml. of ice-cold 25 per cent trichloroacetic acid and the tubes chilled in ice water and centrifuged. Upon 0.5 ml. samples of the supernatant the inorganic phosphorus was determined by means of the method of Fiske and Subbarow as modified by Lohmann and Jendrassik (19). Readings were made with the Klett-Summerson photoelectric colorimeter, the total reaction volume being 5.2 ml. The reproducibility of the duplicate determinations was better than 5 per cent.

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[2] Unpublished experiments.

The homogenates lost about 20 per cent of their Apyrase activity during 6 hours storage in the cold. All assays reported here were started within 50 to 90 minutes from the time of homogenization.

Fig. 1 provides information on the phosphatase activity of normal liver homogenates toward diverse substrates. Details regarding the compounds used can be found in the legend to Fig. 1.

It is obvious from Fig. 1 that there was but negligible activity towards substrates of the alkaline phosphatase, such as β-glycerophosphate and adenosine-3-phosphate. Of compounds that may either be present as impurities of ATP or be formed in the enzymatic breakdown, inorganic pyro-
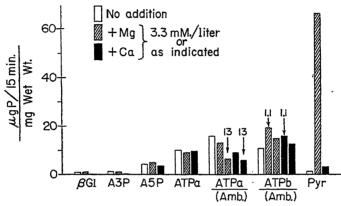
FIG. 1. Hydrolysis of phosphorus esters by liver homogenates from protein-fed rats. Temperature at 37°; pH 7.4. All substrates were employed at concentrations of 3 mM per liter in the form of their sodium salts adjusted to pH 7.4. Pyr., sodium pyrosphosphate (Baker); ATP$_a$, sodium adenosine triphosphate (Rohm and Haas); ATP$_b$, barium adenosine triphosphate (Sigma); A5P, adenosine-5-phosphoric acid (My-B-den, Bischoff); A3P, adenosine-3-phosphoric acid (Schwarz); BGl, sodium β-glycerophosphate (Eastman); Amb., filtered through Amberlite IR-100 (sodium salt, Rohm and Haas).

phosphate was hydrolyzed 3 to 5 times as rapidly as ATP, while adenosine-5-phosphate was split at about one-third to one-fifth the rate of ATP.

The two commercial brands of ATP used were hydrolyzed at different rates. The enzymatic breakdown of Sample ATP$_a$ was not affected by the addition of calcium or magnesium. When solutions of Sample ATP$_a$ were first filtered through the cation exchange resin Amberlite, the rate of hydrolysis in the absence of added cations increased by 50 per cent. The average rates thus obtained exceeded slightly those found by DuBois and Potter (18) at calcium levels of 3 mM per liter, which provided optimal activation of their liver homogenates. With our system, on the contrary, 3 mM of calcium retarded the rate of reaction. We have nevertheless

carried out one set of experiments (Sample ATP_a, Table I) at this calcium level, rather than without added cations, in order to avoid the possibility that differences in the enzyme activity of individual liver homogenates could be due to varying cation levels rather than to changes in the amount of enzyme protein. Small variation of the calcium concentration, about 3 mM per liter, did not significantly alter the rate of hydrolysis.

Sample ATP_b was a commercial barium salt which had been converted into the sodium salt through Amberlite treatment according to the direction of Kielley and Meyerhof (20). With this preparation both magnesium and calcium exhibited stimulatory effects, the former cation being more effective than the latter. The concentration optima were essentially the same for both cations. Experiments in Tables I and II have been carried out with Sample ATP_b in the presence of magnesium chloride, 0.8 mM per liter, which provided optimal activation.

The reasons for the different activity of liver Apyrase towards the two ATP preparations are not clear.[3] Possibly, because Sample ATP_a contained an excess of calcium or magnesium which was only partially removed through Amberlite filtration, the measurements fell on the declining limb of the cation activity curve. At any rate, the results show that not much significance can be attached to the numerical values for Apyrase activity. The relative values obtained with the same ATP preparation on individual organs are significant and reproducible, however.

Rhodanese was determined with the method of Cosby and Sumner (22), adapted to the assay of small homogenate samples. In test-tubes were placed 3 ml. of 0.11 M phosphate buffer (pH 7.4), 0.5 ml. of 0.5 M $Na_2S_2O_3$, homogenate samples corresponding to 10 and 20 mg. of liver tissue from protein-fed and protein-starved rats respectively, and water to make a total volume of 4.5 ml. The tubes were immersed in a water bath at 20°. The reaction was started by the addition of 0.5 ml. of neutralized 0.5 M KCN from a tuberculin syringe and stopped, after 2 minutes, by dumping into the reaction mixture 5 ml. of 10 per cent trichloroacetic acid containing 3.6 ml. of 40 per cent formaldehyde solution per liter. The formaldehyde prevents sulfur formation from thiosulfate. To 3 ml. of clear filtrate, 5 ml. of water and 2 ml. of 5 per cent $Fe(NO_3)_3 \cdot 9H_2O$, containing 5 ml. of concentrated HNO_3 per 100 ml., were added.

For the preparation of blank and standard the filtrate of a tissue sample was used to which cyanide was added after the trichloroacetic acid. One 3 ml. portion, serving as blank, was treated as described above. A second 3 ml. portion received 2 ml. of 0.001 M KSCN and 3 ml. of water

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[3] LePage and Potter (21) recently pointed out that commercial ATP preparations frequently contain trace impurities which inhibit oxygen consumption of tissue homogenates.

before the addition of the iron reagent. 15 minutes after the addition of the iron reagent the color was read in a Klett-Summerson colorimeter. With the procedure outlined above the color intensity follows the Beer-Lambert law.

While 10 to 20 per cent liver homogenates preserve their full rhodanese activity for at least a week when stored in the cold, activity is rapidly lost on greater dilution unless an excess of thiosulfate is present. It is essential, therefore, to add thiosulfate to the reaction tube before addition of the homogenate.

Units—All enzyme units are here expressed in terms of micromoles of assayed reaction product formed per minute under the standard conditions given above. Units per gm. of protein (enzyme concentration) as well as units per total organ per 100 gm. of initial body weight (enzyme content) will be used as measures of the enzymatic activity of liver. The quantity of other tissue constituents is expressed in analogous terms. Values for liver nitrogen were converted into terms of protein by multiplying the difference between total nitrogen and trichloroacetic acid-soluble nitrogen by the factor 6.25. The initial body weights of protein-fed and protein-starved rats were taken as the weights on the day of operation and on the last day on normal stock diet, respectively.

In thirty-eight partially hepatectomized normal rats the ratio of excised liver to total liver averaged 70 per cent, with a standard deviation of ±1.52. Accordingly, the factor 1.43 was used for computing the total liver weight from the weight of the excised portion.

Results

Experimental results concerning the effect of protein starvation on composition and enzymatic activity of liver tissue are summarized in Tables I and II. As in previous studies from this laboratory the majority of assays was performed upon animals depleted for a period of 2 weeks (Group II). The smaller experimental group, No. III, served to explore to what extent protein depletion, when continued almost to the limit of the survival time of the animals, would alter the enzymatic capability of liver tissue. This group included four rats depleted 28 to 35 days, three rats after 44 days of depletion, and four after 49 days.

In Tables I and II mean values of the different quantities under examination are listed, together with the coefficient of variation and the standard error of the mean. To facilitate the evaluation of the results the percentage differences between the group means and their standard errors are also presented so that direction and degree of a change and its significance may be readily seen. From the coefficients of variation it is evident that protein starvation led to an increased heterogeneity of the group.

TABLE I

Concentration of Liver Constituents and Enzymes

Group No.	Type and duration of diet	Designation*	Wet weight / Dry weight	Per 100 gm. wet weight			Per 1 gm. protein		Apryase†	
				Protein	Lipide P	Total lipides	Arginase	Rhodanese	Sample ATP$_a$	Sample ATP$_b$
				gm.	mg.	gm.	units × 10^{-1}	units	units	units
I	18% casein, 7 days	Mean	3.31 (16)‡	18.1 (16)	96 (16)	3.69 (16)	522 (16)	913 (16)	102 (8)	216 (8
		C.v.	±2	±5	±9	±16	±7	±8	±7	±9
		S.e.	±0.014	±0.21	±2.2	±0.149	±9.3	±17.2	±2.4	±7.2
II	Protein-free, 13–17 days	Mean	3.26 (65)	13.3 (65)	73 (65)	5.87 (63)	302 (65)	524 (65)	91 (14)	204 (1
		C.v.	±4	±10	±14	±30	±20	±14	±12	±5
		S.e.	±0.018	±0.16	±1.2	±0.22	±7.6	±9.2	±2.8	±2.9
III	Protein-free, 28–49 days	Mean	3.34 (11)	13.4 (11)	87 (11)	5.82 (10)	213 (11)	520 (11)	106 (2)	178 (3
		C.v.	±7	±13	±20	±28	±31	±9	±2.8	±18
		S.e.	±0.074	±0.53	±6.0	±0.505	±20.2	±13.7	±2.8	±11.6
((II − I)/I)100		% change	−2	−27	−24	+59	−42	−43	−11	−6
		S.e.	±0.7	±1.5	±2.5	±7.2	±2.3	±2.1	±3.7	±3.9
((III − II)/II)100		% change	+2	+1	+19	+1	−30	−1	+16	−13
		S.e.	±2.4	±4.1	±8.4	±10	±7.1	±3.3	±4.3	±5.9

* C.v. = the coefficient of variation = $(s/\bar{x})100$, where $\bar{x}$ is the arithmetic mean and $s = \pm\sqrt{\Sigma(\Delta x)^2/(n-1)}$. S.e. = the standard err of the mean = $s/\sqrt{n} = s_{\bar{x}}$; per cent change = $(\Delta\bar{x}/\bar{x}_1)100$, where $\Delta\bar{x}$ is the difference between the means $\bar{x}_2$ and $\bar{x}_1$; s.e. of per cent chang = $(s_{\Delta\bar{x}}/\bar{x}_1)100$, where $s_{\Delta\bar{x}} = \pm\sqrt{(s_{\bar{x}_1})^2 + (s_{\bar{x}_2})^2}$. The per cent changes as well as the coefficients of variation have been abbreviated to th nearest whole number.

† See the legend to Fig. 1.

‡ The figures in parentheses indicate the number of rats.

The greatest variation was found in the total lipides and arginase levels. This variability of the responses to protein starvation occurred despite the fact that the population was uniform as to strain, sex, and dietary history and carefully selected as to body weight (see Table II). It follows, that comparatively large groups must be employed for the evaluation of dietary effects on composition and enzymatic activity of liver tissue.

Table I deals with the qualitative alterations which result from protein starvation. The data show the pronounced fall of the protein and lipide phosphorus concentration and the concomitant rise of the concentration of the total lipides. Although the rise in total lipides was thus due to an increase in neutral fat, there was but a negligible diminution of the ratio of wet weight over dry weight, corresponding to a reduction of the water concentration from 69.7 to 69.3 per cent. This indicates that reduction of tissue hydration by the accumulation of fat was counteracted by a rise of the glycogen concentration. The occurrence of such a rise has been demonstrated in previous studies from this (3) and other (5) laboratories. Protein and total lipide concentrations remained essentially constant over the entire period of protein depletion studied, while the lipide phosphorus showed a considerable, though statistically not highly significant, increase.

Table I shows, furthermore, that protein depletion led to an altered composition of the liver protein as evidenced by its reduced enzymatic activity and a changed enzyme pattern. After 2 weeks of protein depletion, the arginase and rhodanese concentrations had dropped 42 per cent, while the Apyrase concentration had decreased but little. Contrary to the protein concentration, the enzymatic activity of the liver protein continued to change when the period of protein starvation was extended to 4 to 7 weeks. There was a pronounced fall of the arginase activity, while the rhodanese concentration remained constant. Not much significance can be attached to the observed changes in the Apyrase concentration, since the number of assays is too small. In those livers in which decreased Apyrase concentration was found, the decrease was small in comparison to that of the arginase concentration.

Table II furnishes supplementary information on the net losses in individual constituents and total mass of the liver and in body weight of the animals. The percentage loss in liver protein here recorded for 2 weeks of protein depletion was numerically identical with that previously obtained with a smaller group of depleted animals (3). The excellent reproducibility attests to the usefulness of the protein content as an index of the potentially effective liver mass. This index does not suffice, however, to reveal the full extent of reduction in potential metabolic capability of the organ, since, with 43 per cent of the protein, 68 per cent of both arginase and rhodanese and 50 per cent of the Apyrase activity were

TABLE II

Body Weight, Liver Weight, and Total Protein, Lipide Phosphorus, Lipide, and Enzyme Content of Liver

Group No.*	Designation†	Body weight at operation	Change of initial body weight	Liver weight, per cent initial body weight	Content of total liver per 100 gm. initial body weight					Apyrase	
					Protein	Lipide P	Lipides	Arginase	Rhodanese	Sample ATPa	Sample ATPb
		gm.	*per cent*		*mg.*	*mg.*	*mg.*	*units × 10⁻¹*	*units*	*units*	*units*
I	Mean	279 (16)‡	+6.0§(15)	3.75 (16)	678 (16)	3.62 (16)	138 (16)	355 (16)	621 (16)	67 (8)	155 (8)
	C.v.	±3	±33	±8	±8	±13	±27	±9	±10	±10	±11
	S.e.	±2.3	±0.5	±0.074	±13.4	±0.121	±9.1	±8.1	±15.9	±2.3	±5.6
II	Mean	211 (65)	−19 (65)	2.87 (65)	377 (65)	2.06 (65)	165 (63)	114 (65)	200 (65)	33.5 (14)	76 (11)
	C.v.	±10	±16	±13	±7	±13	±36	±19	±12	±13	±6
	S.e.	±2.5	±0.5	±0.046	±3.2	±0.034	±7.4	±2.7	±3.0	±1.2	±1.2
III	Mean	166 (11)	−34 (11)	2.59 (11)	342 (65)	2.19 (11)	150 (10)	73 (11)	177 (11)	31.4 (2)	60 (3)
	C.v.	±11	±10	±16	±10	±8	±34	±33	±10	±2.6	±27
	S.e.	±5.6	±1.1	±0.122	±10.8	±0.121	±16.2	±7.3	±5.3		±9
((II − I)/I)100	% change	−24		−23	−43	−43	+20	−68	−68	−50	−51
	S.e.	±3.3		±2.3	±2.0	±3.5	±10.6	±2.4	±2.6	±3.9	±3.7
((III − II)/II)100	% change	−21		−10	−9	+6	−9	−36	−12	−6	−21
	S.e.	±2.9		±4.5	±3.0	±6.1	±10.8	±6.9	±3	±9.8	±11.8

* Group I, 7 days on 18 per cent casein diet; Group II, 13 to 17 days on protein-depleted diet; Group III, 28 to 49 days on protein-depleted diet.

† See foot-notes to Table I.

‡ The figures in parentheses indicate the number of rats.

§ Initial body weight here, as in the other groups, is the weight on the last day before transfer of the animals to the semisynthetic experimental diet, whereas for all other data concerning Group I the weight on the day of operation is taken as the initial body weight.

lost. It is of interest to note that the percentage losses due to extension of the period of protein starvation beyond 2 weeks amounted in most instances to about 10 per cent (see (III−II)/II, Table II). Exceptions showing significantly greater losses were the arginase content of the liver and the body mass of the animals. This correlation may possibly be of interpretative significance.

<div align="center">DISCUSSION</div>

It has been noticed by previous investigators (23, 24) that changes in the nutritional state of the animal are paralleled by alterations of the enzymatic activity of liver tissue. Although these observations remain of value in so far as they refer to alterations of the enzyme pattern, they do not permit conclusions as to loss or gain of individual enzyme activities, since no correction had been made for concurrent changes in the composition of the unit mass of tissue which served as the basis of reference. Schultze (25) and Axelrod et al. (26), while studying liver enzymes in nutritional deficiencies, were the first to recognize this limitation of earlier investigations and to demonstrate that the total enzyme content of the organ furnished a better estimate of quantitative changes in enzyme activity.

In recent investigations from several laboratories estimations of the total enzyme content as well as of enzyme concentrations per unit weight of liver protein have been utilized. From the work of Miller (4) and of Schultz (27) on acute inanition it may be deduced that the losses in alkaline phosphatase, cathepsin, and catalase activity are approximately proportional to that of the liver protein, while the losses in xanthine dehydrogenase and cathepsin II (27) are much greater. Published data on complete protein starvation are scanty. Seifter et al. (5) found that D-amino acid oxidase and arginase were lost more rapidly than the liver protein. Benditt et al. (6) reported that in chronic protein deficiency the alkaline phosphatase content of the total liver remained constant, while cytochrome oxidase and succinoxidase decreased at a somewhat faster rate than the total liver protein.

The 30 to 40 per cent of liver protein that is rapidly lost during the initial stages of inanition or protein starvation is usually designated "labile" liver protein. Kosterlitz (28) has presented experimental evidence indicating that the labile protein arises from the breakdown of the cytoplasm of the parenchymatous cells. The author refers to the easily lost portion of the cytoplasm as "labile" cytoplasm. It is obvious that enzymes situated mainly in the "labile" cytoplasm will display a greater percentage loss than the total protein, those evenly distributed between labile and "stable"

cell components will decrease proportionally to the total protein, while the quantity of enzymes residing only in stable regions will remain constant.

From the data quoted from the literature as well as from our own results it appears that enzymes such as xanthine dehydrogenase, D-amino acid oxidase, rhodanese, and arginase, which are concentrated particularly in liver tissue and, presumably, mainly cytoplasmic constituents of parenchymatous cells, are lost more rapidly than the total liver protein. Phosphatases, on the other hand, which are detectable in cytoplasm and nucleus of parenchymatous and non-parenchymatous cells, decrease proportionally to or less than the total liver protein. It would seem, therefore, that the differential loss of individual enzymes roughly parallels their distribution between "labile" and "stable" cellular entities. Conversely, the percentage net losses may furnish an estimate as to what proportion of a given entity is lost in nutritional deficiencies. If, for instance, we assume that rhodanese and arginase are localized exclusively in the cytoplasm of the liver cells, then the data in Table II would indicate that 68 per cent of the cytoplasm was lost during 2 weeks of protein depletion. It is of interest to note that these enzyme assays fail to reveal any qualitative difference between the "labile" and the residual "stable" cytoplasm, since the ratio of rhodanese to arginase activity in the tissue from rats depleted of protein for 2 weeks was identical with that obtained in the casein-fed series. While this is in keeping with the results of chemical analyses of cytoplasmic constituents (28), conclusive evidence would require comparative assays upon a greater variety of enzymes and firmer knowledge as to their distribution.

There are indications that "lability" of cell constituents may not be an inherent chemical characteristic, but may rather be conditioned by the metabolic state of the organism. In Miller's experiment (4) on acute inanition the alkaline phosphatase content of the liver decreased proportionally to the protein, while according to Benditt prolonged restriction of dietary protein resulted in similarly great losses of liver protein but failed to reduce the phosphatase content of the organ. The functional difference between both states of protein depletion apparently lies in the type and quantity of substrates which are metabolized by the liver cells. Our own experiments have shown that in advanced stages of complete protein starvation arginase activity diminished faster than rhodanese activity or total liver protein. It is possible that the degree of saturation with substrate is responsible for the differential preservation of enzyme activity under various conditions of protein depletion. Evidence in support of this view will be presented in a forthcoming paper.

SUMMARY

After a 2 week period of protein depletion of rats the livers had lost 43 per cent of their protein and lipide phosphorus, whereas the amount of total lipides remained fairly constant. The loss of 68 per cent in total arginase and rhodanese activity of the organ greatly exceeded that in total protein, while the reduction in adenosine pyrophosphatase activity of 50 per cent more nearly approached the protein loss.

Extension of the period of depletion up to 4 to 7 weeks resulted in additional small losses of the order of 10 per cent in most liver components, with the exception of the total arginase activity, which decreased an additional 30 per cent.

The significance of the differential losses in enzyme activity has been discussed.

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THE EGG WHITE INHIBITOR OF INFLUENZA VIRUS HEMAGGLUTINATION

II. ELECTRON MICROSCOPY OF THE INHIBITOR*

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PLATES 1 AND 2

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The results of studies on the purification and characterization of the egg white inhibitor of influenza virus hemagglutination have been described in recent reports (1–3). Viscosity measurements (1, 2) indicated that inhibitory activity was associated with a highly asymmetric component.[1] Preliminary examination of purified inhibitor with the electron microscope failed to provide unequivocal support for this supposition; however, beaded, thread-like structures were occasionally observed in some preparations (2). Recently, more detailed electron micrographic examinations have been made of a series of inhibitor preparations. The results, which bring the electron micrographic data into close accord with the viscosity findings, are reported in the present paper.

Materials and Methods

The studies reported here were made with preparations of the purified inhibitor and with precipitates obtained on exposure of the inhibitor preparations to an electric current (3) in the Tiselius cell. Attempts were made to obtain micrographs of the thick portion of egg white diluted with 0.06 M phosphate buffer of pH 7.2, of the thick egg white dialyzed against 0.06 M phosphate buffer of pH 7.2, and of the gel-like material sedimented from purified preparations by spinning at 67,000 × g (2) in the air-driven ultracentrifuge. None of the latter materials provided data of significance to the problem of the character of the inhibitor.

Semipurified inhibitor was prepared from thick egg white by the phosphate buffer method previously described (2). In some instances, the material was reprecipitated several times. The properties of the preparations employed in the present work are shown in Table I.

Electrically precipitated inhibitor (3) was obtained from Preparation A236-PEII (Table I). Samples were submitted to preliminary dialysis in the cold against phosphate buffers of pH 7.2 and ionic strength 0.2, 0.1, 0.05, or 0.01. Precipitate which formed during dialysis against the more dilute buffers (Table II) was removed by low speed centrifugation. The supernatants were placed in the descending limb and cap of an 11 ml. Tiselius cell and subjected to 7.6, 7.2, 6.7, or 4.0 volts per cm. at 0.2, 0.1, 0.05, or 0.01 ionic strength, respectively. A ground glass appearance was seen within 10 minutes; within 1 to 2 hours, precipitate which formed throughout the descending limb and at the bottom of the ascending limb had settled into the cap. The material in the cap (volume about 4 ml.) was collected apart from the material in the two limbs and centrifuged at 1000 $\times$ g (2000 r.p.m.) for 10 minutes. The supernatant fluid was carefully pipetted from the water-clear, gel-like sediment, of which a

TABLE I

Semipurified Inhibitor Preparations Employed for Electron Microscopy

Preparation	N per ml.	Purification factor
	γ	
A236-PEII.................................	187	29
A253-PEI..................................	256	29
A253-PEIII................................	38	42
A178-PIII-EI*.............................	148	41

* Preparation described previously (2).

volume varying from about 0.1 to 1.0 ml. was obtained from 11 ml. of starting material. The yields and properties of the precipitates obtained in the Tiselius cell are given in Table II.

For *electron micrography*, the semipurified preparations (in 0.06 M phosphate buffer of pH 7.2) were diluted with water. Dilutions of the gel-like pellets obtained in the Tiselius cell were made with NH_4OH-NH_4Cl buffer of pH 8.6 and ionic strength 0.1. The materials were deposited on collodion membranes prepared in the usual way. The films were washed with distilled water after they were dry and were shadowed lightly with chromium at an angle of 15°. An RCA type B instrument was employed for making the micrographs.

The method for assay of *inhibitory activity*, with heated swine influenza virus and washed chicken erythrocytes, has been previously described (2).

Results

In the present work, it was found that filamentous structures could be demonstrated without difficulty in inhibitor preparations of high concen-

tration. In order, however, to account for the extremely high specific viscosity, it was necessary to demonstrate definite particle asymmetry in solutions of inhibitor at the low concentrations, 8 to 80 γ of N per ml., employed in the viscosity studies. This was difficult because at least one dimension of the particles is exceedingly small, and the significant structures, though shadowed with metal, were invisible on the fluorescent screen of the electron microscope. Numerous micrographic exposures were therefore made at random, the proper focus being sought with the aid of images of larger extraneous particles, such as salt deposits and dust particles.

TABLE II

Properties of Fractions Obtained by Dialysis of Inhibitor Preparation A236-PEII* in Phosphate Buffers at pH 7.2 and by Electrophoresis of Dialyzed and Centrifuged Material

Ionic strength of buffer	Origin of fraction	Ppt.				Supernatant
		Volume	Inhibition titer†	Nitrogen†	Purification factor	Purification factor
		ml.		*mg. per ml.*		
0.20	Dialysis	0				30‡
	Electrophoresis	1.0	101,000	0.453	67	
0.10	Dialysis	0				30
	Electrophoresis	0.5§	144,000	0.646	67	
0.05	Dialysis	1.2	34,000	0.420	24	29
	Electrophoresis	0.8	85,000	0.552	46	
0.01	Dialysis	1.5	101,000	0.875	34	17
	Electrophoresis	0.1				

* Inhibition titer 18,000; 0.187 mg. of N per ml.; purification factor 29, referred to whole egg white. Approximately 15 ml. were dialyzed against each buffer.
† Refers to undiluted fraction.
‡ Expected value.
§ Volume low as result of accident during collection.

Furthermore, an appearance of filamentous structure is shown by the collodion membrane after it is coated with metal. This is illustrated, Fig. 1, in the micrograph of a film on which polystyrene latex particles[2] were deposited for purposes of size comparison. The possible interference of such structure in the background with interpretation of the character of the particles in inhibitor solutions is revealed in Fig. 2, which is a micrograph of the edge of a droplet of inhibitor. The region at A is covered with a thin layer of inhibitor which grades sharply into a region[3] of aggre-

[2] These particles, prepared by The Dow Chemical Company, Midland, Michigan, were supplied by Dr. Robley C. Williams. Their mean diameter is about 253 mμ.
[3] The high contrast and unusual appearance of the aggregates are due to the presence of calcium salts.

gation, *B*. From micrographs such as those of Figs. 1 and 2, it is evident that many of the difficulties in demonstrating filaments in dilute preparations may be due to inability to distinguish between scattered filaments of inhibitor and the background of the shadowed film. Similar problems of interpretation have been encountered by other investigators (4-6).

The obviously filamentous structures in concentrated and relatively highly purified inhibitor solutions are seen in the micrograph of electrically precipitated inhibitor in Fig. 3. For this, the precipitate[4] obtained at 0.2 ionic strength, Table II, was diluted 1:3.5 with NH_4OH-NH_4Cl buffer and spread on the film. In the micrograph, there are seen irregular masses of various sizes consisting, apparently, of filamentous structures oriented nearly in parallel. The filaments in this micrograph are imperfectly resolved because of the depth of the preparation, but exhibit, nevertheless, an irregular, nodose structure which suggests linear aggregations of spheroidal units. This appearance, which could have been caused by a number of factors, including motion of the screen during exposure of the plate or astigmatic artifacts, has been seen in many other pictures of the material in concentrated preparations.

The occurrence of filaments in dilute solutions of the inhibitor has been established in preparations such as the one of Fig. 4. This was Preparation A253-PEIII, Table I, diluted 1:10 with water, and placed on the collodion film containing 3.8 γ of N per ml. In this micrograph there are filamentous structures differing unmistakably from the structure shown by the metal-shadowed collodion film illustrated in Fig. 1. The degree to which the film structure in the background (Fig. 1) affects the interpretation of the finer structures of the inhibitor seen in Fig. 4 cannot be determined. The large fibrils, of about 10 mμ maximum width, are variable in length and are irregularly curved. Some appear to taper at the ends, which merge into the background, and there is the appearance of branching. The resolving power of the microscope was not great enough to show

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[4] As previously described (3), voluminous, gelatinous precipitates frequently develop on electrophoresis of solutions of either crude egg white or semipurified egg white inhibitor. These precipitates, recovered from the Tiselius cell as described above and previously (3), contain much of the inhibitory activity of the starting solution and thus provide inhibitor of relatively high purity. The results of electrophoresis of Preparation A236-PEII at ionic strengths 0.2 to 0.01, Table II, show that the largest amount of precipitate was obtained at 0.2 ionic strength. The purification factor was 67, twice as great as the factor of the dialyzed material, 30. An identical purification factor was observed with the precipitate obtained at 0.1 ionic strength. Precipitates less satisfactory with respect to volume or degree of purity were obtained at 0.05 and 0.01 ionic strengths. It will be observed in Table II, also, that precipitates were obtained by dialysis alone at 0.05 and 0.01 ionic strengths. These precipitates, however, were of little, if any, greater purity than the dialyzed solutions.

clearly an elementary structure within the larger fibrils. In all pictures of such material, however, the filaments give the appearance of consisting of chains of spheroidal particles of about 5 mμ diameter, measured where the fibrils taper into the background. There was no evidence of a predominant length of the fibrils.

The micrograph of Preparation A178-PIII-EI after dilution to 1.48 γ of N per ml. is shown in Fig. 5. This is the same preparation that yielded the micrographs of Figs. 1 and 2 of the previous publication (2); the micrograph of Fig. 5 was found in a further study of the negatives previously taken. Because this picture is somewhat out of focus, the collodion background is obscured; nevertheless, there is seen a profusion of fibers appearing to interlace on the film.

DISCUSSION

The electron micrographs obtained in the present study demonstrate that semipurified preparations of the egg white inhibitor of influenza virus hemagglutination contain a filamentous component. The existence of such a component in native egg white may be inferred from certain simple macroscopic observations. For the present purposes, egg white may be described as consisting of three distinct layers of albumen arranged concentrically about the yolk. These are the outer thin, the thick, and the inner thin layers. The two thin layers, which have a relatively low viscosity, may be drawn readily into a pipette and they form drops with ease. In contrast, the thick layer, which is essentially a thick walled bag containing the inner thin albumen and the yolk, is highly viscous and coherent, with the result that it is pipetted with difficulty, and, when allowed to fall from a pipette, shows a pronounced tendency to form strings rather than spheroidal drops. Moreover, once the thick albumen has been started into a pipette, it is possible to collect most, if not all, of this layer in a single, continuous operation with the pipette raised a cm. or so above the albumen surface. Portions of the thick layer, located initially at considerable distance from the tip of the pipette, may be drawn in before other portions located just slightly to the side of the tip, as if the whole layer were a single, thick strand with complete longitudinal, but limited lateral, continuity. Since this property of the thick albumen is easily destroyed by gentle mechanical stirring, it would appear that the native thick layer is permeated by a fibrous mesh, which imparts to the thick white its property of coherence. When the thick white is bathed in 0.06 M phosphate buffer of pH 5.7, a pH at which the inhibitor is precipitated, and is agitated on a rocking device, fibers are formed which exhibit beautiful birefringence in polarized light. Electron microscopy of such fibers revealed only gross fibrils in which the character of the fundamental units could not be discerned.

For the following reasons, it would appear that the inhibitory activity of native egg white and of semipurified preparations of inhibitor is closely associated with filamentous structures such as those observed in the microscope: (a) of the several albumen layers, the most viscous is the thick; this layer possesses also the highest inhibitory activity (2); (b) as purification of the inhibitor proceeds, the inhibitor and the viscous component move together (1–3); (c) both inhibitory activity and viscosity are reduced by treatment with influenza virus, but not with virus inactivated by heating (1);[1] (d) the dimensions of the inhibitor, as inferred from sedimentation and viscosity studies (2), are in rough agreement with those obtained for the filamentous particles by electron micrography; and (e) when the inhibitor is sedimented (2) in high centrifugal fields, the pellets obtained are voluminous, transparent, and gelatinous; from the last property, it may be supposed that the pellets, just as the thick white, owe their structural character to a fibrous element.   For these reasons, it is permissible to conclude, at least tentatively, that the inhibitor is identical (cf. (2, 7, 8)) with the mucoprotein ovomucoid-β (9), also known as ovomucin, the acknowledged structural protein of egg white (for references and discussion see Hoover (10)); however, it is not certain that the filamentous character, per se, is requisite for inhibitory activity.   The dimensions both of the filaments observed in inhibitor preparations (Figs. 2 to 5) and of the constituent spheroidal units of the filaments are variable.   The latter vary from about 5 to 10 mμ in diameter.   As can be seen from the micrographs, it is not possible to discern a predominant filament length.   A possible mechanism for the formation of fibrils from the filaments is suggested by the micrograph (Fig. 3) of the thick gel-like material obtained by electrical precipitation in the Tiselius cell.   In this micrograph, the filaments are seen to be aligned in parallel, possibly as the result of an orienting effect of the electric current (3).

Electron micrographs of purified inhibitor preparations resemble closely those of fibrinogen preparations shown by Hall (5) and, to a lesser extent, the electron micrographs of F-actin reported by Rozsa, Szent-Györgyi, and Wyckoff (6).   The filaments of inhibitor preparations in electron micrographs in good focus have a nodose or beaded appearance of a character that might be expected if the filaments consisted of linear aggregates of spheroidal particles of 5 to 10 mμ diameter.   Suggestions that some filamentous proteins may arise by linear arrangement of spheroidal elementary units have been seen in the electron micrographic study of G- and F-actin (6) and of the transformation of fibrinogen to fibrin (11).   While both spheroidal and filamentous structures were seen in the egg white preparations, the possible relationship between the two types of structures is obscure.

SUMMARY

Electron micrographic studies were made on semipurified preparations of the egg white inhibitor of influenza virus hemagglutination and on the gel-like material precipitated from such preparations in an electrical field in the Tiselius cell. Particulate structures of highly asymmetric shape, in the form of filaments, fibrils, or fibers, were observed both in concentrated solutions of the electrically precipitated gel and in dilute preparations not precipitated in the Tiselius cell. The filaments were oriented in parallel in the electrically precipitated material but were distributed at random in dilute preparations. The filaments appeared to be linear aggregates of spheroidal particles and varied in width from 5 to 10 mμ. No evidence was seen of a predominant length of filaments or fibers. The demonstration of asymmetry of particle shape was in accord with the previously demonstrated extremely high viscosity of egg white inhibitor preparations. Evidence for the identity of the asymmetric particulate material, probably ovomucoid-β, with the inhibitor of influenza virus hemagglutination is discussed.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Electron micrograph of polystyrene latex particles (Batch 580G, The Dow Chemical Company, Midland, Michigan) lying on a clean collodion film. Magnification, 56,000 ×.

FIG. 2. Edge of droplet of egg white inhibitor (Preparation A253-PEI) showing: *A*, inhibitor spread on collodion film; *B*, agglomerates of inhibitor; and *C*, an area of clean collodion film free of inhibitor. Magnification, 28,000 ×.

PLATE 2

FIG. 3. Thick layer of electrically precipitated inhibitor formed from Preparation A236-PEII. Magnification, 56,000 ×.

FIG. 4. Inhibitor, Preparation A253-PEIII (purification factor 42), diluted to 3.8 γ of N per ml. The gross white images are deposits of phosphate buffer salts. Magnification, 56,000 ×.

FIG. 5. Inhibitor, Preparation A178-PIII-EI (purification factor 41), diluted to 1.48 γ of N per ml. Magnification, 56,000 ×.
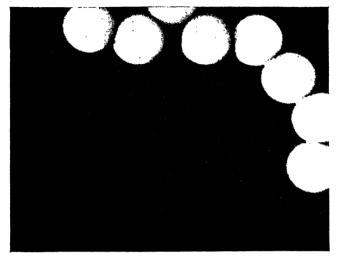
FIG. 1
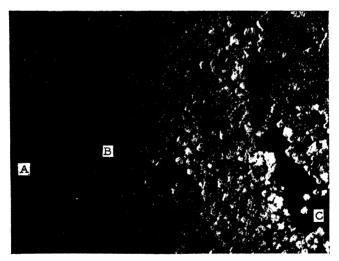
FIG. 2

(Sharp, Lanni, and Beard: Influenza virus hemagglutination. II)
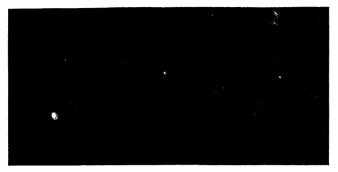
Fig. 3

Fig. 4

Fig. 5

(Sharp, Lanni, and Beard: Influenza virus hemagglutination. II)

THE ENZYMATIC FORMATION OF CITRIC ACID STUDIED WITH C14-LABELED OXALACETATE*

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Citric acid has come to be regarded as being on a side-path in the Krebs cycle. This view is based on the results of isotope experiments which were widely interpreted as being inconsistent with the presence of a symmetrical tricarboxylic acid in the main line of reactions (1–5). Ogston, however, has recently pointed to the possibility that citrate in an enzyme-substrate complex may behave as an asymmetrical molecule (6) and Potter and Heidelberger have reported experiments (7) in support of Ogston's theoretical considerations. These workers isolated radioactive citric acid formed by a rat liver homogenate in the presence of $C^{14}O_2$ and non-isotopic oxalacetate and pyruvate. On oxidation of this citrate with rat liver homogenate, radioactive α-ketoglutarate was obtained in which practically all the activity resided in the α-carboxyl group. Since the two carboxyl groups of α-ketoglutarate correspond to the primary carboxyl groups of citrate, which are symmetrical in the free acid, it was concluded from the foregoing result that citrate was degraded asymmetrically by the enzyme, in accordance with Ogston's proposal.

Removal of objections to citrate as an intermediate in the main line of reactions of the tricarboxylic acid cycle reopens the question of the place to be assigned to it in the cycle. The recent report of Stern and Ochoa (8) indicates that citrate itself may be the initial condensation product. These authors used aged extracts of acetone-dried pigeon liver and demonstrated citrate formation from acetate and oxalacetate as substrates. Adenosine triphosphate (ATP) was found to be necessary, suggesting a phosphorylated intermediate. The enzyme preparations employed were very low in aconitase activity, indicating that the citrate did not arise via

isocitrate or *cis*-aconitate but was, presumably, the initial product of the reaction. For simplicity this reaction may be represented as follows:

(*a*)   Acetate

$$CH_3 \cdot COOH$$

$$+$$

$$\overset{*}{C}OOH \cdot CH_2 \cdot CO \cdot COOH \longrightarrow \overset{*}{C}OOH \cdot CH_2 \cdot COH \cdot COOH$$

Oxalacetate   Citrate

with the $CH_2 \cdot COOH$ group shown to the upper right:

$$CH_2 \cdot COOH$$

The present study with oxalacetate labeled with C^{14} in the β-carboxyl group (a relatively small amount of activity was also contained in the α-carboxyl group) was undertaken to test this formulation of the condensation reaction. The reaction, as written, demands that the resulting citrate contain little or no isotope in the tertiary carboxyl group and none in the non-carboxyl carbons, but that it be labeled in a primary carboxyl group as illustrated by equation (*a*), in which the asterisks indicate the location of the isotope. Furthermore, α-ketoglutarate, formed by enzymatic degradation of the citrate of equation (*a*), should contain isotope solely in the α-carboxyl group, in accordance with Ogston's thesis and the observations of Potter and Heidelberger, and as illustrated by equation (*b*).

(*b*) $CH_2 \cdot COOH$ $CH_2 \cdot COOH$

$$\overset{*}{C}OOH \cdot CH_2 \cdot COH \cdot COOH \rightleftarrows \overset{*}{C}OOH \cdot CO \cdot CH_2 + CO_2 \longrightarrow$$

Citrate α-Ketoglutarate

$$COOH \cdot CH_2 \cdot CH_2 \cdot COOH + \overset{*}{C}O_2$$

Succinate

The intermediate steps linking citrate and α-ketoglutarate have been omitted.

It has been noted previously (5, 8) that participation of a phosphorylated derivative of acetic acid in the condensation reaction could lead to the formation of an asymmetrical phosphate ester of citric acid. However, the isolation of free citric acid prior to enzymatic degradation to α-ketoglutarate necessitates a postulate such as Ogston's to explain the presence of label exclusively in the α-carboxyl group of the resulting α-ketoglutarate. It will be apparent from inspection of equations (*a*) and (*b*) that, if the initial condensation reaction involved the carbon corresponding to the methylene group of the original oxalacetate, the resulting tricarboxylic acid would be labeled in the tertiary carboxyl group, and the α-ketoglutarate derived therefrom would be unlabeled.

The results of the present study are in complete accord with the demands of the reaction mechanism postulated for citrate formation with

acetate and oxalacetate as substrates (equation (a)) and confirm and extend the observations of Potter and Heidelberger offered in support of Ogston's proposal.

Procedure

C^{14}-Oxalacetate was prepared enzymatically and isolated by partition chromatography. An aliquot was degraded to determine the distribution of the isotope, and the balance of the oxalacetate, together with non-isotopic acetate, was used for the enzymatic preparation of isotopic citric acid. The resulting citrate, following the addition of non-isotopic carrier, was isolated by partition chromatography and the quinidine derivative was prepared. An aliquot of the quinidine citrate was degraded chemi-

TABLE I

Distribution of Isotope in C^{14}-Oxalacetate Used in Citrate Formation

Degradation fraction	Activity of sample assayed in counts per min. per mM carbon (a)	Dilution factor* (b)	Activity of undiluted oxalacetate in counts per min. per mM β- or α-COOH carbon† (a × b)
β-COOH...............................	39,100	28	1.1 × 10⁶
α-COOH...............................	1,330‡	28	3.7 × 10⁴

* 0.017 mM of C^{14}-oxalacetate + 0.46 mM of carrier.

† These are the values found in the oxalacetate recovered after incubation with the citrate-forming enzyme. They are not significantly different from those determined prior to incubation.

‡ This represents a maximum value, since the present method of degradation results in a small amount of contamination of the α-COOH by the β-COOH group.

cally and free citric acid was recovered from the remainder. This isotopic citrate was used to study the enzymatic oxidation of citric acid to α-ketoglutarate. The resulting α-ketoglutarate, following the addition of non-isotopic carrier, was isolated by partition chromatography, and the semicarbazone was prepared and degraded chemically. Some of the isotopic citrate was also used in a study of the reversibility of the citrate-forming reaction, as will be described.

Experimental details will be presented in a later section.

RESULTS AND DISCUSSION

In Table I are presented the results of the degradation of the C^{14}-oxalacetate. It will be noted that the β-carboxyl group contains at least 30 times as much isotope as the α-carboxyl group. The residual oxalacetate, isolated by partition chromatography following incubation with the citrate-

forming enzyme, was found to be essentially unchanged with regard to level and distribution of isotope. It was of potential importance to know whether or not the oxalacetate had been altered during citrate formation, since, if appreciable randomization of isotope had occurred between the two carboxyl groups during the incubation period, it would have been necessary to know its extent in order to evaluate properly the results of the subsequent citrate degradation.

The results of the chemical degradation of the C14-citrate are presented in Table II. The procedures employed yielded three separate fractions containing the primary carboxyl groups, the tertiary carboxyl, and the non-carboxyl carbons, respectively. Detectable activity occurred only in the primary carboxyl groups.

TABLE II

Distribution of Isotope in Various Fractions from Chemical Degradation of C14-Citrate

Degradation fraction	Activity of sample in counts per min. per mM compound assayed (a)	Dilution factor (b)	Activity in undiluted citrate in counts per min. per mM citrate (a × b)
Primary carboxyls.........................	4340	153*	6.6×10^5
Tertiary carboxyl..........................	0	76.5†	0
Non-carboxyl carbon (Hg-acetone complex).	0	76.5†	0

* 4 mg. of C14-citrate + 149 mg. of carrier for isolation; 1:1 dilution with carrier quinidine citrate for degradation; 2 mM of primary COOH per mM of citrate; $((4 + 149)/4) \times 2 \times 2 = 153$.

† 1 mM of tertiary COOH or Hg-acetone complex per mM of citrate; otherwise as above; $153/4 \times 2 = 76.5$.

The results of the enzymatic oxidation of C14-citrate to α-ketoglutarate are given in Table III. The CO_2 evolved in this step contains the tertiary carboxyl group of the citrate. The slight activity[1] noted in this fraction is consistent with the presence of a small amount of isotope in the α-carboxyl group of the original oxalacetate. However, it may have arisen from the enzymatic degradation of some of the α-ketoglutarate to succinate and $C^{14}O_2$. That the enzymatic oxidation of citrate did not proceed beyond α-ketoglutarate to a significant degree is indicated by the appearance of only a trace of activity in the CO_2 evolved during the oxidation.

[1] The actual determination, made on 23 mg. of $BaCO_3$, was 2 c.p.m. above background. This figure has no quantitative significance and can only be taken to indicate that a trace of activity was present in this fraction. Assuming that this activity was present in the tertiary carboxyl of the citrate, the failure to detect activity in the tertiary carboxyl group obtained by chemical degradation of the citrate (Table II) may be accounted for by dilution owing to the use of non-isotopic carrier in the chemical degradation.

The chemical degradation of the α-ketoglutarate produced by enzymatic oxidation of the citrate effects a separation of the primary carboxyl groups of the citrate. The CO_2, derived from the α-carboxyl of the α-ketoglutarate, represents one of these primary carboxyl groups, while the other is contained in the succinate which results from the chemical decarboxylation of the α-ketoglutarate (see equation (b)). The presence of activity only in the CO_2 fraction (Table III) indicates clearly that the isotope was not randomized between the primary carboxyl groups of the citrate and that these groups are not interchangeable in the course of the enzymatic oxida-

TABLE III

Distribution of Isotope in CO_2 and α-Ketoglutarate from Enzymatic Oxidation of C^{14}-Citrate

Degradation fraction	Activity of sample in counts per min. per mм compound assayed (a)	Dilution factor (b)	Activity in undiluted citrate in counts per min. per mм citrate (a × b)
CO_2 from enzymatic oxidation of C^{14}-citrate.	32*	38†	1.2×10^3
CO_2 from $KMnO_4$ oxidation of C^{14}-α-ketoglutarate semicarbazone (α-carboxyl of α-ketoglutarate).................	920	430‡	3.9×10^5
Succinate from $KMnO_4$ oxidation of C^{14}-α-ketoglutarate semicarbazone..............	0	215§	0

* See foot-note 1.

† This factor is based on the assumption that the CO_2 comes entirely from the tertiary carboxyl group of citrate. C^{14}-Citrate diluted 4:153; 153/4 = 38 (see Table II).

‡ C^{14}-Citrate diluted 4:153; 0.067 mм of C^{14}-α-ketoglutarate + 0.31 mм of carrier for isolation; 1:1 dilution with CO_2 from the semicarbazide for degradation; (153/4) + (0.377/0.067) × 2 = 430.

§ (153/4) × (0.377/0.067) = 215.

tion of citrate. The fact that this result was obtained after the citrate had been isolated, converted to the quinidine derivative, recovered as the free acid, and then incubated with the enzyme system is a compelling argument against an asymmetrical derivative, such as citryl phosphate, being responsible for the difference in behavior of the primary carboxyl groups.

In comparing the results in Tables I to III, it will be noted that the level of activity decreases from oxalacetate to citrate to α-ketoglutarate. This is probably due, in part, to dilution by endogenous substrate at the enzymatic steps. In addition, the dilution factors calculated for the various steps at which non-isotopic carrier material was added are probably accurate only to within 10 per cent, because of the errors involved in the vari-

ous chemical determinations. Regardless of these considerations, the results indicate quite clearly that isotope from the β-carboxyl group of the oxalacetate has entered that primary carboxyl group of citrate from which the α-carboxyl carbon of the α-ketoglutarate is derived.

An experiment to test the reversibility of the citrate-forming reaction was carried out by incubating non-isotopic oxalacetate and labeled citrate with the same enzyme system which was used to prepare the isotopic citrate. The residual oxalacetate, isolated and degraded at the end of the run, contained no activity, indicating that the reaction was not reversible under the conditions prevailing. This negative result cannot be considered as conclusive proof of irreversibility of the reaction, since conditions may not have been such as to favor the reversal.

EXPERIMENTAL

Preparation and Isolation of Labeled Oxalacetate—Labeled oxalacetate was prepared by incubating oxalacetate (9) in the presence of $NaHC^{14}O_3$ with an extract obtained from an acetone powder of pigeon liver, according to the general procedure of Utter and Wood (10) with some modifications.[2] After dialysis against 0.9 per cent KCl for 6 hours, the aqueous extract of the acetone powder was lyophilized. Three Warburg vessels were set up as follows: an enzyme solution corresponding to 60 mg. of the lyophilized extract was placed in one side arm and 1.1 mM of $NaHC^{14}O_3$ containing approximately 2.25×10^7 c.p.m. were placed in the other side arm, while the main chamber contained (expressed as final molarity) 0.002 M NaATP, 0.07 M oxalacetate (neutralized to pH 6.4 with Na_3PO_4), 0.002 M $MnCl_2$, and 0.01 M cysteine. The total volume was 2.1 ml. The vessels were incubated for 20 minutes at 28° after gassing with nitrogen. After addition of 1 mM of additional oxalacetate, the combined cup contents were deproteinated by acidifying to a concentration of 3 N H_2SO_4 and centrifuging. After washing the precipitate once, the combined supernatants were mixed with 2 weights of Johns-Manville Celite (No. 535). The Celite was placed on a large sintered glass funnel and oxalacetic acid was recovered by washing four times with peroxide-free ether. After evaporation of the ether under a stream of dry air at room temperature, the small aqueous residue was lyophilized. The recovery of oxalacetate by this extraction method is about 80 per cent.

The lyophilized oxalacetate was subjected to chromatography on silica gel according to the method of Isherwood (11), with some modifications. A 1 inch column was prepared with 40 gm. of silicic acid (Merck) and 3.0 N H_2SO_4 as the immobile phase. The stronger acid was used to reduce the spontaneous decarboxylation of oxalacetate. The chromatogram was de-

[2] Utter, M. F., unpublished data.

veloped with 10 and 15 per cent n-butanol in chloroform, with thymol blue[3] as an external indicator. Acids more mobile than oxalacetate (e.g. pyruvate) were removed in about 135 ml. of 10 per cent butanol, after which point elution with 15 per cent butanol was begun. The oxalacetate was obtained in the 115 to 220 ml. fraction. 0.5 mM of carrier oxalacetate was added to this fraction and the acid was extracted by shaking with dilute NaOH. The aqueous phase was freed from butanol and chloroform by shaking twice with small portions of ether. Recovery of known oxalacetate, chromatographed as described, is about 90 per cent. The free acid was regained by acidifying to a concentration of 3 N H_2SO_4 and repeating the Celite extraction as previously described. The aqueous residue, held at $0°$, was neutralized just before use. Aliquots were removed for the determination of oxalacetate and radioactivity. Oxalacetate was determined by decarboxylation with Al^{+++} (12), which also served as the degradation procedure for obtaining the β-carboxyl group. For obtaining the α-carboxyl carbon of the pyruvate fragment, Krebs and Johnson's adaptation (13) of the ceric sulfate oxidation of Fromageot and Desnuelle (14) was used.

The two steps were carried out in sequence; a standard Warburg vessel was used and the CO_2 collected in NaOH in the center well without the usual fluted papers. The alkali was washed from the center well with dilute NaOH, converted to $BaCO_3$, and plated on a filter paper disk for counting, in a fashion similar to that described by Henriques et al. (15). The data on the radioactivity of the oxalacetate are given in Table I.

Preparation and Isolation of Labeled Citrate—The acetone powder from pigeon liver used for the formation of the citrate was prepared according to Kaplan and Lipmann (16). For use, 1 gm. of the powder was extracted with 10 ml. of 0.02 M phosphate buffer (pH 7.8) and centrifuged. The supernatant was stored on solid CO_2 overnight, held at $28°$ for 4 hours, and centrifuged again and the precipitate was discarded. 4.0 ml. of the supernatant were placed in each of the side arms of a 150 ml. Warburg vessel. The main chamber contained the following in a total volume of 26 ml.: 1.17 mM of Na oxalacetate (1.1 × 10^{6} c.p.m. per mM), 0.06 mM of NaATP, 0.08 mM of $MgCl_2$, 0.2 mM of Na acetate, 1 mM of potassium phosphate (pH 6.95), 0.1 mM of neutralized cysteine, and 320 mg. of crude coenzyme A (16).[4] Also present in the main chamber was 0.43 mM of

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[3] The indicator used with the column made up with 3 N H_2SO_4 was 0.005 N with respect to NaOH. When 0.5 N H_2SO_4 was used as the immobile phase, as in the isolation of citrate and α-ketoglutarate, the indicator was made 0.002 N with respect to NaOH.

[4] The coenzyme A was incubated for 1 hour at $28°$ in 0.1 N NaOH before use to destroy the alkali-labile oxidized form of diphosphopyridine nucleotide and TPN presumably present in the crude preparation, and thus prevent equilibration of

pyruvate from spontaneous breakdown of oxalacetate which occurred sub-
sequent to silica gel fractionation. After gassing with nitrogen, the vessel
was incubated for 30 minutes at 28°, a period found to be optimum in a
series of preliminary trials.

After removal of aliquots for determination of citrate (17) and for oxal-
acetate degradation and assay, 149 mg. of carrier citrate were added to the
incubation mixture which already contained about 4 mg. of isotopic citrate
according to analysis. The mixture was deproteinated by adding H_2SO_4.
After centrifugation, the supernatant was neutralized and reduced in vol-
ume on a steam bath. After addition of H_2SO_4 to a concentration of 3 N,
2 weights of Celite were mixed with the solution, and the resulting powder
was subjected to continuous ether extraction for 30 hours. After evapora-
tion of the ether, the aqueous residue was lyophilized and chromatographed
on a silica gel column, with 0.5 N H_2SO_4 as the immobile phase and 30 and
35 per cent n-butanol in chloroform as eluting agents. After approximately
500 ml. of the 30 per cent butanol had passed through the column, the
citric acid was eluted with the 35 per cent solvent. The citric acid ap-
peared in the 250 to 750 ml. fraction in this experiment. In our hands,
the behavior of citric acid on the silica gel column is less predictable than
that of the more readily eluted acids.

The citrate was recovered from the solvent by extracting successively
three times with water. The combined water extracts were reduced in
volume and steam-distilled to remove butanol. Citrate, determined on
an aliquot, was recovered in an over-all yield of 95 per cent from the
extraction and chromatographic procedures. After reduction in volume,
a portion containing 0.21 mM of citrate was stored in a frozen state for
subsequent use in testing the reversibility of the condensation reaction.
The remaining citrate was recovered as the quinidine salt (18, 5). After
recrystallization, the quinidine salt melted at 125–129° (uncorrected), as
did an authentic sample. The mixed melting point was not depressed.
This finding alone is not a conclusive criterion of purity since a large
amount of carrier had been added. However, the results of the various
isolations and degradations, when considered together, leave no doubt that
the activity actually resided in the citrate and that its location in the
molecule has been reliably indicated.

Chemical Degradation of C^{14}-Citrate—The primary and tertiary carboxyl
groups of citrate were obtained as separate fractions by submitting an ali-
quot of the quinidine citrate to the procedure of Weinhouse *et al.* (5). The

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oxalacetate with the symmetrical dicarboxylic acids. Such equilibration would
lead to randomization of the isotope between the carboxyl groups of the oxalacetate.
The solution was brought to pH 7.0 with HCl before placing in the vessel.

tertiary carboxyl, which is obtained as CO, was converted to CO_2 (19) to permit counting as $BaCO_3$. The non-carboxyl carbons were obtained as the Hg-acetone complex from another aliquot of the quinidine citrate by the method of Van Slyke (20). The acetone was liberated from the complex by boiling in 1 N HCl, removed by steam distillation, and reprecipitated as the Hg complex, in which form it was counted. The activities of the various fractions are given in Table II.

Enzymatic Oxidation of C^{14}-Citrate to α-Ketoglutarate—After preliminary studies, the following procedure was adopted for the conversion of citrate to α-ketoglutarate. The enzyme preparation was that described in the preparation of labeled oxalacetate except that it was not dialyzed and lyophilized. 2.5 ml. of the enzyme solution were placed in the side arm of a 150 ml. Warburg vessel containing the following substances in the main chamber in a total volume of 5.75 ml.: 0.11 mM of isotopic Na citrate recovered from the quinidine citrate (5), 0.125 mM of potassium phosphate (pH 7.4), 15 mg. of triphosphopyridine nucleotide (TPN) with a purity of about 40 per cent (21), 0.01 mM of $MnCl_2$, and 1 mg. of riboflavin (dissolved in 0.5 ml. of 0.005 M NaOH). Riboflavin was added to permit non-enzymatic reoxidation of $TPN \cdot H_2$ (22). The center well contained a fluted filter paper saturated with 10 N NaOH. The vessel was incubated at 38° with air as the gas phase for 130 minutes, at which time oxygen uptake was nearly complete and α-ketoglutarate accumulation, as judged by prior runs, at a maximum. The alkali was recovered from the center well and its carbonate converted to $BaCO_3$ for measurement of radioactivity. Determination of α-ketoglutarate (23) on an aliquot indicated about a 60 per cent conversion of the citrate. After 0.31 mM of carrier α-ketoglutarate had been added, deproteinization and ether extraction were carried out approximately as for the oxalacetate. The ether was evaporated, the residue dried by lyophilizing, and chromatographic separation carried out on a silica gel column made up with 0.5 N H_2SO_4. For developing the column 10 per cent butanol in chloroform was used. Under these circumstances α-ketoglutarate appears after about 400 ml. of effluent have been collected and is removed from the column in approximately the next 300 ml., about 90 per cent of the total recovery coming off in the last half of the fraction. In the present run, 0.28 mM of α-ketoglutarate determined colorimetrically (23) was recovered in the fraction from 567 to 742 ml. The α-ketoglutarate was extracted by titrating with dilute NaOH and the aqueous phase was washed with several small portions of ether. The volume was reduced by lyophilizing and the semicarbazone was prepared and recrystallized twice. This melted at 210° (uncorrected), as did the derivative prepared directly from the reagents. The melting point of a mixture of the two was not depressed. 0.17 mM of

α-ketoglutarate semicarbazone was then degraded to CO_2 and succinate by use of $KMnO_4$ (4). The succinate was removed from the reaction mixture by continuous ether extraction, isolated as the silver salt (4), and counted as such. The activities of the various fractions are given in Table III.

Test of Reversibility of Condensation Reaction—This experiment was set up and conducted in a manner similar to that for citrate formation, with the addition of 0.21 mM of C^{14}-citrate, prepared as described previously. 0.04 M malonate was added to prevent the formation of labeled oxalacetate via the oxidative reactions of the Krebs cycle. The added oxalacetate was, of course, non-isotopic. The residual oxalacetate remaining after the incubation period was isolated and degraded as already described and found to contain no activity. A control run, conducted simultaneously with aliquots of all the materials employed in the experimental cup, except that citrate was omitted, showed active citrate formation.

All measurements of radioactivity were made with a conventional end window Geiger-Muller tube with an efficiency of approximately 10 per cent under the circumstances obtaining. All samples were converted to $BaCO_3$ for radioactivity assay, except for the silver succinate and Hg-acetone complex which were counted as such. Active samples were counted at "finite" thickness (10 to 25 mg. of $BaCO_3$ on an area of 2.85 sq. cm.) and corrected for self-absorption. The over-all error of the radioactivity assay is in the neighborhood of ± 3 per cent.

SUMMARY

The distribution of isotope in citrate formed enzymatically from C^{14}-labeled oxalacetate ($C^{14}OOH \cdot CH_2 \cdot CO \cdot COOH$) and non-isotopic acetate has been studied. Chemical degradation of the C^{14}-citrate revealed a significant concentration of isotope only in the fraction containing the primary carboxyls of the citrate molecule, in consonance with a reaction mechanism involving linkage of the methyl group of acetate at the carbonyl group of oxalacetate. Enzymatic oxidation of the C^{14}-citrate yielded α-ketoglutarate with labeling exclusively in the α-carboxyl group. This fact indicates that the isotope from the β-carboxyl group of oxalacetate occurred in only one of the primary carboxyls of the citrate. Thus the metabolic behavior of citrate is not that predicted from the structural symmetry which is apparent when the molecule is considered as an isolated chemical entity, but is in accord with the recent proposals of Ogston (6), as reported previously by Potter and Heidelberger (7). The present study extends the observations of the latter workers to another species of animal. In addition, the use in the present work of labeled oxalacetate rather than CO_2 for the preparation of labeled citrate, coupled with a

more detailed degradation of the citrate molecule, makes available critical evidence in support of the postulated citrate-forming reaction not provided by the prior work.

These developments make it clear that it is possible for citrate to lie on the main line of reactions of the tricarboxylic acid cycle, as originally proposed by Krebs, and, in conjunction with the recent report of Stern and Ochoa (8), point to citrate rather than *cis*-aconitate or isocitrate as the initial condensation product in the cycle. What evidence the present study brings to bear on the question indicates that under the conditions employed, the citrate-forming reaction is not reversible.

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MEASUREMENT OF AMYLASE ACTIVITY*

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The Nelson photoelectric method for the determination of glucose in biological fluids (1) has proved to be the method of choice in this laboratory and in many others, owing to its reproducibility, stability of color, and excellent agreement with the Beer-Lambert law. Nelson, however, states that the method is unsuitable for the determination of diastatic activity. Since it is convenient and economical for a laboratory running large numbers of glucose and amylase determinations to use the same reagents for reduction and color development, it seemed advisable to attempt to modify the amylase procedure to this end.

The difficulty in using the substrate of the Somogyi amylase method (2) with the Nelson reagents arises from the clouding of the final colored solution by the presence of unhydrolyzed starch. It was reasoned that, if a smaller amount of starch were present, the final solution might be clear. This was realized by diluting the Somogyi substrate after incubation and running the diluted solution through the Nelson procedure. Thus, while the original relative concentrations of substrate and serum which Somogyi found to be optimum were preserved, the final colored solutions were clear in all cases, including the starch blank, and were suitable for photoelectric reading. This final concentration of starch may be attained in a slightly different manner by using only 0.3 per cent starch and 0.2 ml. of serum and omitting the 1:5 dilution after incubation. It is interesting to note in this regard that "micromethods" in current use (2) require the employment of 1 ml. of serum.

Macromethod

Reagents—These are the same reagents which are used in the Somogyi saccharogenic method (2): 1.5 per cent starch paste;[1] 1 per cent NaCl con-

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[1] The starch paste was prepared in the usual manner by triturating 1.5 gm. of Baker's soluble starch powder "according to Lintner" in a small amount of cold distilled water, pouring the suspension into 100 ml. of boiling distilled water, boiling for a minute, and stoppering the flask before cooling at room temperature. The 0.3 per cent starch paste for the micromethod was prepared in the same manner.

taining 3.0 ml. of 0.1 N HCl; 5 per cent $CuSO_4 \cdot 5H_2O$; 6 per cent sodium tungstate.

Procedure—5.0 ml. of starch, 2.0 ml. of acidified saline, and 1 ml. of serum are incubated at 40° for 30 minutes in Tube A. A control, Tube C, is incubated containing the same solutions but substituting 1 ml. of water for the serum. A third tube, Tube B, is set up containing 5.0 ml. of water, 1 ml. of serum, and 2.0 ml. of acidified saline. This tube is for the determination of serum glucose and is not incubated. At the end of the 30 minute period 1 ml. of copper sulfate followed by 1 ml. of sodium tungstate is added to each of the three tubes. The tubes are shaken, then centrifuged

TABLE I

Comparison of Methods

Results expressed as "glucose equivalents," *i.e.* $A + (B - C)$.

Specimen No.	Folin-Wu*	Nelson (micro)	Nelson (macro)
1	88	82	
2	142	147	
3	64	65	
4	48	53	
5	71	74	75
6		66	68
7†	1089	1162	1035
8	103	102	93
9	62	58	58
10†	690	748	701

* Read on a Duboscq colorimeter.

† This was artificially prepared "high" by adding a small amount of saliva to the serum. In this case an additional dilution of the substrate was necessary in all three methods before reduction.

or filtered. To 1 ml. of each filtrate or supernatant add 4 ml. of water. 1 ml. of each of these diluted filtrates is now subjected to the Nelson procedure. The per cent transmissions are read at 660 mμ, and their values in mg. per cent of glucose are read from a previously standardized table.[2]

Calculation—

$$\text{Glucose equivalents} = A + (B - C)$$

where A, B, and C represent the concentrations in mg. per cent of glucose in the respective tubes and the amylase value is expressed in glucose equivalents.

[2] The Coleman junior spectrophotometer, model 6A, was used for the photometric measurements.

Micromethod

Reagents—0.3 per cent starch paste; NaCl (acidified) (see macromethod) 1 per cent CuSO$_4$·5H$_2$O; 1.2 per cent sodium tungstate.

Procedure—The procedure, with the above dilute reagents, is the same as for the macromethod with the following exceptions: (1) use 0.2 ml. of serum and 0.8 ml. of water in Tube A; (2) use 0.2 ml. of serum and 5.8 ml. of water in Tube B; (3) use 1 ml. of the filtrate directly for the reduction without dilution.

Transmissions are read at 660 mμ and the same table of glucose equivalents is used as for the macromethod, since the absolute amounts of reducing substances are the same.

TABLE II

Reproducibility of Micromethod

Results expressed as "glucose equivalents."

Specimen No.	1st determination	2nd determination
10	60	60
11	56	57
12	142	140
13	103	103
14*	769	763
15*	860	820
16*	1650	1630

* Artificial "high" prepared by adding saliva to serum. Dilution of the substrate was necessary before reduction.

Results

Random specimens of human sera submitted for amylase assay were run by both the old method (Folin-Wu reduction) (3) and the method described above. In all cases separate incubations were made. Table I records amylase activity in glucose equivalents. The greatest difference noted between the two methods was 10 per cent.

Reproducibility of Micromethod—Table II records amylase activity on separately incubated samples of identical serum specimens, with use of the micromethod. In seven consecutive determinations, the greatest difference that occurred was less than 5 per cent.

SUMMARY

1. A procedure has been presented for the determination of amylase activity in biological fluids by the Nelson blood glucose method.

2. A procedure for a micromethod for serum amylase assay has been outlined.

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THE SURFACE CHEMISTRY OF BONE

I. RECRYSTALLIZATION*

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The problem of the skeletal deposition of toxic metals has received considerable attention. One common observation has been the localization of the foreign mineral in areas of active calcification (1–6). This phenomenon has led to an association of the calcification process with the mechanism of deposition of non-physiological minerals in the skeleton. Since the phenomenon of bone growth is poorly understood, the situation did not lend itself to further investigation.

With the finding that uranium (2, 7, 8), radiophosphate (9, 10), and fluoride[1] are all taken up by bone by ionic exchange, a physicochemical process unrelated to cellular activity, a new emphasis has been placed on the surface chemistry of bone. It was hoped that the concentration of foreign or labeled minerals in regions of active calcification could be explained in part by a greater surface activity of bone taken from these areas. The experiments performed did not critically test this hypothesis. Rather, they demonstrated that fresh unashed bone undergoes recrystallization at a surprising rate. However, a means of testing bone preparations for surface activity, even in the presence of active recrystallization, was devised.

Methods

Unless otherwise indicated, bone specimens were prepared and phosphate exchange was determined as reported previously (8) by an equilibration of powdered bone *in vitro* at pH 7.3 with 0.0013 M phosphate buffer containing P^{32}. The exchangeable (surface) phosphate is determined by isotope dilution. In all cases, phosphate was determined by the method of Fiske and Subbarow (11).

Results

Comparison of Amounts of Exchangeable Phosphate of Newly Deposited and Established Bone—Femora were taken from several young (4 to 6 week-old) and adult (1½ year-old) chinchilla rabbits, and the following

portions separated for exchange studies: (a) from young femora, newly deposited metaphyseal bone obtained by dissecting approximately 3 mm. from the distal surface of the metaphysis of the distal end of femur, (b) also from young femora, newly deposited periosteal bone obtained by scraping approximately 1 mm. from the surfaces of the shaft after removal of the periosteum, and (c) from adult femora compact bone obtained from the interior of shaft wall after 2 to 3 mm. of both the internal and external surfaces had been removed by grinding.

One-half of each of the three fractions was ashed in alkaline glycol (12). The other half was defatted (1:1 alcohol-ether for 24 hours) and dried. All samples were ground to a particle size between 60 and 120 mesh. The resulting six specimens were then tested for phosphate exchange by equilibration for 48 hours in radiophosphate buffer. The averaged results of duplicate determinations are presented in Table I.

TABLE I

Exchangeable Phosphate of Bone Taken from Different Parts of Femur

Bone fraction	Exchangeable phosphate*	
	Fresh	Ashed
	per cent	*per cent*
Subperiosteum....................................	50.4	12.7
Metaphysis.......................................	29.3	13.0
Compact shaft....................................	9.2	13.4

* Obtained after 48 hours of equilibration.

It is apparent from these data that the ashing procedure seriously modified the nature of the bone mineral. The exchange of phosphate in new bone from the periosteal and metaphyseal areas was markedly reduced, while that of compact shaft was increased as a result of ashing, but all came to the same per cent exchange.

As expected, fresh bone taken from actively growing areas showed much greater surface activity than did that taken from the stable non-growing shaft. However, the magnitude of the exchangeable fraction of the periosteal bone was unbelievably large. An apatite crystal could not possibly have half of its phosphate groups in the surface (13).

It is possible that the greater proportion of exchangeable phosphate in the fresh, newly calcified preparations is due to the presence of a greater proportion of organic phosphate. Periosteal bone, for example, contains twice as much organic material as does adult, compact shaft. Calculations show that some 30 per cent of the total phosphate is removed from periosteal bone by ashing in alkaline glycol. This compares with only a 4 per cent loss in the case of compact shaft. Technical difficulties have, as yet, prevented a direct test of the exchangeability of the organic phos-

phate in demineralized bone. A representative ester, sodium α-glycerophosphate, however, was shown not to undergo exchange.[1]

A reinvestigation of the behavior of *fresh* bone when placed in contact with phosphate buffer seemed necessary. Previous studies had been made, for the most part, with bone that had been glycol-ashed. In the few studies made on fresh bone (8) only short periods of exposure to radioactive buffer were used.

Comparison of Exchangeable Phosphate of Fresh and Ashed Bone As Function of Time—The earlier work (10) on glycol-ashed bone was repeated, and the time of equilibration extended to 18 days. The results are

FIG. 1.
FIG. 2.
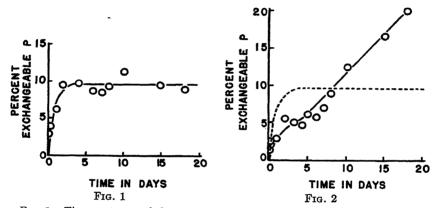
FIG. 1. The constancy of the exchangeable phosphate of ashed bone with time.
FIG. 2. The increase in the exchangeable phosphate of fresh bone with time. The dotted line represents comparable data obtained with ashed bone. Note that a temporary plateau is observed from the 2nd to 6th day, in agreement with earlier studies (8).

presented in Fig. 1. As before (10), a "quasi-equilibrium" was attained in 2 days, with little or no further exchange over the remaining 16 day period.

When fresh bone (dried and defatted) taken from the femur shafts of rabbits was studied over a similar period of time, as seen in Fig. 2, the fraction of phosphate calculated as exchangeable did not approach a steady state but continued to rise, reaching a value on the 18th day which was twice that obtained for ashed bone.[2] Ionic exchange is a rapid process.

[1] Neuman, M. W., unpublished results.
[2] The equation for calculating exchangeable phosphate is based on the assumption that the system is at equilibrium. In any case in which equilibrium is not reached, the values for exchangeable phosphate are admittedly in error. They are sufficiently accurate, we believe, for comparative purposes and a new calculation based on kinetic considerations has been developed (see foot-note 3).

It would be expected that a state of equilibrium would be reached in less than 2 days. It was evident that the concept of ionic exchange was not adequate to explain the behavior of *fresh* bone when exposed to phosphate buffer.

The most reasonable explanation was that fresh bone, unlike ash, underwent a process of recrystallization. It is known that heat treatment results in crystal growth and a more stable crystal lattice. A minimal amount of recrystallization is therefore to be expected with heat-treated bone. It remained to be established that *fresh* bone does undergo recrystallization.

Effect of Temperature on Exchange of Phosphate by Fresh Bone—With bone preparations incapable of recrystallization, the final distribution of the isotope between bone and solution depends only on the relative amount of surface. Under such circumstances, altering the temperature of equi-
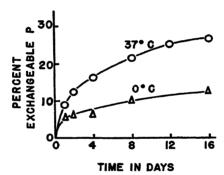
FIG. 3.   The effect of temperature on the rate of phosphate exchange in fresh bone.

libration should affect only the rate at which equilibrium is attained. The final distribution of isotope should be independent of temperature. When recrystallization takes place, however, isotope is continually incorporated into the reforming crystals and equilibrium is not reached during the experiment (6 to 18 days). Therefore, it would be expected that the interaction of *fresh* bone and radiophosphate buffer would be markedly affected by temperature variations. Increased temperature should increase the rate of recrystallization and the rate of incorporation of isotope.

The exchange of radiophosphate with glycol-ashed bone (which should recrystallize slowly if at all) has been reported to be independent of the temperature of equilibration (10). Further study has given less consistent results. While many ashed preparations attain a state of equilibrium independent of temperature, others do not. None of the ashed preparations, however, shows the marked dependence on temperature that is invariably observed with fresh bone samples. Typical results are given in Fig. 3. Obviously no equilibrium state has been attained.[2]

Lack of Reversibility of Fresh Bone-Buffer System—An ionic exchange process should be completely reversible. The relative distribution of radiophosphate between the solid phase and the solution should be approximately the same, regardless of whether the P^{32} was in the solid or the solution initially. Or, stated in another way, that fraction of phosphate calculated as exchangeable should be the same whether determined by "adsorption" or "desorption" methods. As described earlier, the ashed bone-buffer system showed such a reversibility (10). These data have been recalculated and presented in Fig. 4 for purposes of comparison.

Fresh bone samples were equilibrated for 12 days at 37° with radiophosphate, at which time the buffer was replaced with inert phosphate and
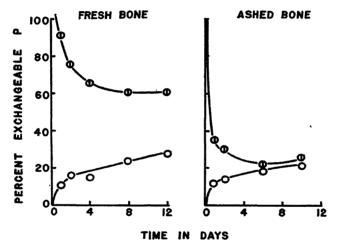
FIG. 4. Data illustrating the lack of reversibility of the fresh bone-buffer system. Data showing the reversibility of the ashed bone-buffer system are taken from a previous publication (10). In each case, the lower curve (O) represents adsorption data; the upper curve (⊕) desorption.

further equilibrated for an additional 12 days. The results are also presented in Fig. 4 and indicate a marked lack of reversibility. This was the expected result if recrystallization had taken place. Radiophosphate "buried" in the interior of the newly reformed crystals would not be available for exchange in the desorption period. Reversibility could be observed only if the equilibration period were at least 6 months in duration (time for *complete* recrystallization).

Specific Activity-Time Relationships—Thus far, the data indicate that fresh bone, in contact with radiophosphate buffer, undergoes ionic exchange and recrystallization. Is there any means by which these two processes might be studied separately?

From mathematical consideration[3] it can be predicted that the logarithm of the specific activity (SA) of the radiophosphate buffer should fall linearly with time as a result of either ionic exchange or recrystallization. It seemed possible that experimental conditions could be so arranged that the rates of reaction of the two processes would be sufficiently different to give SA-time curves capable of analysis.

Preliminary experiments showed that at elevated temperatures the SA-time curve was indeed biphasic. Typical results, obtained at 37°, are presented in Fig. 5. The first linear portion of the curve may be attributed to the sum of ionic exchange *and* recrystallization; the second portion with less slope may be assigned to recrystallization. Extrapolation of the recrystallization curve to zero time gives an intercept value from
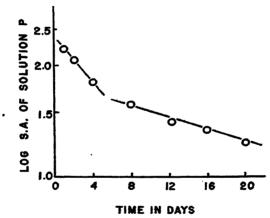
FIG. 5. The separation of ionic exchange and recrystallization by a semilogarithmic plot of SA *versus* time.

which a fairly accurate estimation of the surface phosphate may be calculated. In this case, a value of about 8.6 per cent is obtained for fresh powdered shaft. From the slope of the recrystallization curve one may

[3] The equation relating the rate of change of the specific activity (SA) of solution with time may be derived from mass law considerations. The rate at which isotope will pass from solution to the bone is proportional to the SA of the solution (SA_s). The rate at which isotope returns to the solution is proportional to the SA of the exchangeable phosphate of bone (SA_b). Thus, $(dSA_s)/(dt) = K(SA_s - SA_b)$. If one assumes that the rate of recrystallization is a linear function of time over the experimental period, this relation may be substituted and integrated to give $k_1\ln(k_1SA_s - k_2) = k_3t$, where k is a constant. For this relation to be valid, the temperature, the volumes, and the phosphate concentrations of both the solid and liquid phases must all remain constant. This relation applies to change in SA_s resulting from *both* ionic exchange and recrystallization.

estimate the rate at which this process takes place. In this case, the average half life of a crystal of powdered shaft was of the order of 40 days.

Thus, a method is available for the separate estimation of both the exchange and recrystallization rates of fresh bone.

DISCUSSION

Many years ago, Hevesy proposed that bone mineral undergoes recrystallization *in vivo*. He believes, however, that the process is one of dissolution and reprecipitation resulting from variations in the concentrations of calcium and phosphate in the tissue fluids (14). The results reported here would indicate that bone mineral undergoes a recrystallization even in solutions of constant composition, and the rate at which this phenomenon takes place makes one wonder at the stability of the skeleton.

The physiological importance of the recrystallization phenomenon cannot be stated in view of our present, limited knowledge. It is clear, however, that data on skeletal distribution obtained from the administration of radioactive calcium and phosphate to animals are seriously complicated by the diversity of mechanisms by which these ions may be fixed by the skeleton, *i.e.*, accretion, ionic exchange, and recrystallization.

Regardless of the apparent stability of bone *in vivo*, the calcified structures must be considered very labile and sensitive from the chemical point of view. The manipulative techniques employed in the study of bone must be chosen with caution. For example, ashing procedures, while removing organic material, cause a marked alteration in the structure and nature of the bone mineral and should be used only in certain types of experiments. At the moment, it seems virtually impossible that the organic matrix may be removed from fresh bone without causing a serious modification in the mineral substance.

SUMMARY

The behavior of powdered, fresh bone when suspended in phosphate buffer has been studied. In solutions of constant composition, the bone mineral was found to undergo a rapid recrystallization.

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THE EFFECT OF HIGH PRESSURES ON PEPSIN AND CHYMOTRYPSINOGEN*

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A study of the inactivation of trypsin and chymotrypsin by high pressures was reported previously (1). The effects of high pressure on pepsin and chymotrypsinogen, and on the activation of chymotrypsinogen by trypsin, have now been examined.

The effect of high pressure on crystalline pepsin was investigated by Matthews, Dow, and Anderson (2), in particular as affected by magnitude of pressure and duration of pressing. Most of their experiments were carried out at 35.5° and pH 4.8, at one concentration, and with pressing times of 1 to 8 hours. In the present investigation with pepsin, the effect of pH was studied in detail, and the effects of concentration, magnitude of pressure, pressing time, and multiple application of pressure were investigated in solutions in several buffers. The solutions were pressed at 25° in all cases, and generally for only 5 minutes.

Pepsin was found to be considerably more sensitive to pressure than was trypsin or chymotrypsin. It was most stable at pH 3.5 to 4.0. Under a given set of conditions, increasing the concentration of the pepsin generally resulted in a somewhat lower percentage loss of activity, whereas the reverse was true with trypsin. Increase in either pressure or pressing time resulted in further loss of activity of pepsin under all conditions tested; there was no tendency for the loss of activity to reach a maximum short of complete inactivation.

Pressure did not activate chymotrypsinogen. As measured by subsequent activation by trypsin, chymotrypsinogen was found to be somewhat more stable under high pressures than was trypsin or chymotrypsin. The behavior was similar except that chymotrypsinogen was not significantly affected by.multiple applications of pressure.

A number of papers have been published on the effect of pressure on reactions catalyzed by enzymes (3–11). Fränkel and Meldolesi (12) reported that the activity of pepsin, trypsin, and diastase was increased by pressures up to 10 atmospheres. Benthaus (13) found that the digestion

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of gelatin by pepsin was retarded by a pressure of 1500 atmospheres (1520 bars), but returned to normal on release of the pressure.

The activation of chymotrypsinogen by trypsin was partly inhibited by pressures as low as 1000 bars; this inhibition was practically complete at 3000 bars. At this point, however, after release of the pressure, the activation proceeded at practically the same rate as in the freshly prepared control. Further increase in pressure resulted in less activation following release of the pressure, until practically no subsequent activation occurred after pressing at 5700 bars.

<div align="center">EXPERIMENTAL</div>

Apparatus and Materials—The apparatus used for the pressure experiments was that described previously (1). A commercial preparation of crystalline pepsin was used.[1] Crystalline chymotrypsinogen was prepared from beef pancreas by the method of Kunitz and Northrop (14), and was then dialyzed against distilled water until salt-free and finally lyophilized. The crystalline trypsin sample used was obtained from Dr. Moses Kunitz of The Rockefeller Institute for Medical Research.

<div align="center">*Effect of Pressure on Pepsin*</div>

Procedure—The procedure employed was similar to that previously described (1). For the experiments on the influence of pH, pepsin was dissolved in distilled water and the pH adjusted to the desired point with dilute acid or alkali. For all other experiments, pepsin was dissolved in one of the following 0.1 M buffers: pH 1.9 chloride, pH 4.0 phthalate, pH 4.0 acetate, or pH 5.9 phosphate. The pressing time was 5 minutes for all experiments except those on the effect of duration of pressing; the temperature of the pressed solutions was 25°. The concentration of pepsin used was 0.05 mg. of protein nitrogen per ml. for all experiments except those on the influence of concentration. Pepsin was assayed by the hemoglobin method of Anson (15).

Influences of pH and Concentration—Pepsin was practically completely inactivated at 7600 bars at pH 2.0 to 5.2. A series was then run at 6100 bars over the pH range 1.5 to 6.5. As shown in Fig. 1, the maximum retention of activity was at pH 3.5 to 4.0.

Runs similar to those above were also made in solutions in the four buffers. All of the values except those obtained with phthalate buffer at pH 4.0 were close to the curve in Fig. 1; the value in the latter was only 32 per cent. Results were consistently lower in the phthalate buffer than in the acetate buffer.

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[1] Purchased from the Armour Laboratories, Armour and Company, Chicago, Illinois.

Three series of runs, each employing four different concentrations of pepsin, were made as follows: pH 1.9 chloride buffer at 5500 bars, and pH 4.0 acetate and phthalate buffers at 6100 bars. The results as given in Table I show the highest percentage retention of activity in all cases at the highest concentration.
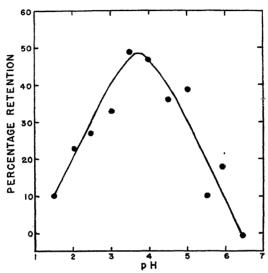
FIG. 1. The influence of pH on the effect of pressure on pepsin. Pressure, 6100 bars; time, 5 minutes; concentration, 0.05 mg. of protein nitrogen per ml.

TABLE I

Influence of Concentration on Effect of Pressure on Pepsin (Pressing Time, 5 Minutes)

Protein nitrogen	Per cent retention of activity		
	Chloride buffer, pH 1.9, 5500 bars	Acetate buffer, pH 4.0, 6100 bars	Phthalate buffer, pH 4.0, 6100 bars
mg. per ml.			
0.15	53	59	45
0.05	50	51	32
0.015	36	50	29
0.005	38	48	33

Effect of Magnitude of Pressure—The effect of magnitude of pressure was tested on pepsin solutions in three buffers. The results as given in Fig. 2 show that the curves are roughly parallel, except in buffer of pH 1.9 above 6000 bars. Other than the latter, no tendency was found for a portion of the activity to be resistant to pressure.

Effects of Duration of Pressing and of Multiple Pressing—Pressing times of 5, 15, and 60 minutes were compared for pepsin solutions at 5500 bars in chloride buffer at pH 1.9, and at 6100 bars in acetate buffer of pH 4.0. The results as given in Table II show an increase in inactivation with increase in pressing time.
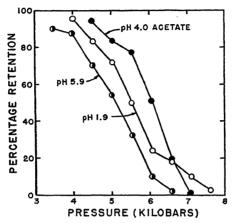
FIG. 2. The effect of pressure on pepsin. Pressing time, 5 minutes; concentration, 0.05 mg. of protein nitrogen per ml.

TABLE II

Effects of Duration of Pressing and Multiple Application of Pressure on Pepsin
Concentration, 0.05 mg. of protein nitrogen per ml.

Duration of pressing	Per cent retention of activity	
	Buffer, pH 1.9, 5500 bars	Acetate buffer, pH 4.0, 6100 bars
min.		
5	50	51
15	28	28
60	10	2
5 (3 times)	14	11
Calculated	13	13

Two experiments were performed in which solutions of pepsin in chloride buffer at pH 1.9 and acetate at pH 4.0 were pressed three times for 5 minutes each at 5500 and 6100 bars, respectively, with intervening periods of 5 minutes each when the pressure was lowered to 400 bars. The results (Table II) in both cases were much lower than those for a single 15 minute pressing at the same pressure. On the assumption that the same percentage of pepsin was inactivated during each 5 minute pressing as in the initial 5 minute period, the total amount of inactivation to be

expected was calculated (Table II) and found to be in good agreement with the experimental value.

The solutions at pH values below 4.5 at the higher pressures and concentrations used formed turbidities or precipitates on pressing in all cases. This was not the case at or below pH 4.5.

Effect of Pressure on Chymotrypsinogen

Procedure—The procedure used was essentially that previously described (1), except for the treatment with trypsin before assay. The chymotrypsinogen solutions used generally contained 1 mg. of protein nitrogen per ml. 1 ml. of the pressed solution or control was mixed with 3 ml. of 0.1 M phosphate buffer, pH 7.6, 1 ml. of trypsin solution (containing 0.00067 mg. of protein nitrogen) was added, and the mixture allowed to stand at 25° (14). 1 ml. aliquots were removed for assay after 2 hours, and sometimes also after 24 hours, and added to 4 ml. of M/15 hydrochloric acid to stop the action of trypsin. Aliquots of this solution were assayed immediately by the method of Anson (15). Under the above conditions the activity produced by trypsin treatment for 2 hours was about half of that for 24 hours. The 2 hour results were used in calculating the percentage retention of activity of the pressed sample compared with the corresponding control.

An experiment was performed in which the trypsin treatment was begun (a) as soon as possible after the pressing ended (about 15 minutes), and (b) after an interval of 2 hours. The results checked closely (69 and 68 per cent, respectively, of the activity of the control), indicating that the change in chymotrypsinogen induced by pressure was permanent.

Non-Activation by Pressure—In a number of experiments, the activities of the pressed and control samples were measured before addition of the trypsin. A very small amount of activity was found in all cases, but there was no significant difference in the activity of the pressed and control samples.

Effects of Duration of Pressing and Magnitude of Pressure—Solutions of chymotrypsinogen (1 mg. of protein nitrogen per ml.) in distilled water were pressed for 5 and 60 minutes, respectively, at 7500 bars. After treatment with trypsin for 2 hours, the pressed samples had 65 and 63 per cent, respectively, as much activity as the corresponding controls.

Solutions of chymotrypsinogen (1 mg. of protein nitrogen per ml.) in distilled water and in 0.1 M phosphate buffer, pH 7.6, were pressed for 5 minutes at pressures of 6700, 7500 and 8400 bars, respectively. In each case the results at 7500 and 8400 bars were in good agreement; the retention of activity at 6700 bars in both cases was considerably higher than those at 7500 bars.

The above results show that with chymotrypsinogen, like chymotrypsin

and trypsin but unlike pepsin, further increases in magnitude or pressing time beyond a certain level have little or no effect.

Effects of Concentration and pH—Solutions of chymotrypsinogen in distilled water containing 0.34, 1.0, 3.3, and 10.2 mg. of protein nitrogen per ml., respectively, were pressed at 7600 bars for 5 minutes. The retention of activity after 2 hour trypsin treatment was 90, 62, 50, and 37 per cent, respectively, showing that an increase in concentration results in a decrease in per cent retention of activity after trypsin treatment.

Three experiments were carried out in which the pH of the solution was 3.1, 5.6, and 7.6, respectively, the concentration of chymotrypsinogen being 1 mg. of protein nitrogen per ml. After pressing 5 minutes at 7600 bars, and subsequent 2 hours trypsin treatment, the per cent retention of activity was 92, 62, and 41, respectively, showing decreasing stability under pressure with increasing pH in the range 3.1 to 7.6.

Effect of Multiple Applications of Pressure—A solution of chymotrypsinogen (1 mg. of protein nitrogen per ml.) in distilled water was pressed successively for 5 minutes each at 7600, 400, 7600, 400, and 7600 bars. After trypsin treatment for 2 hours, the activity was 69 per cent of that of the control. In a similar experiment in which the pressure was applied only once at 7600 bars for 5 minutes, the corresponding value was 68 per cent. Another similar solution was pressed for 5 minutes at 7600 bars, the pressure released, the solution allowed to stand 22 hours at 25°, and then again pressed at 7600 bars for 5 minutes. The activity after 2 hours trypsin treatment was 69 per cent of that of the control.

Similar experiments with solutions of chymotrypsinogen in 0.1 M phosphate buffer, pH 7.6, gave values of 40 and 34 per cent, respectively, for single and triple applications of pressure. Considerable amounts of precipitate formed in the pressed solutions in both cases. The above results show that multiple application of pressure had little or no effect on chymotrypsinogen.

Isolation of Crystalline Chymotrypsinogen after Pressing—A solution of 1 gm. of chymotrypsinogen in 20 ml. of distilled water was pressed for 5 minutes at 5100 bars. The activity after a 2 hour trypsin treatment was 99 per cent of that of the control. On precipitation by ammonium sulfate and recrystallization, very long needles[2] characteristic of chymotrypsinogen were obtained.

In a second similar experiment, on pressing for 5 minutes at 7500 bars some fluorescing material was formed. On attempting to recrystallize the

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[2] On recrystallization of chymotrypsinogen from solutions somewhat more than 25 per cent saturated with ammonium sulfate, much shorter crystals sometimes appear which are shaped somewhat like a canoe as viewed from above. These crystals form much more slowly than the characteristic long needles.

material, over half of the total protein separated in an amorphous form of low activity after trypsin treatment. A fraction was finally obtained from the filtrate, which on recrystallization formed the very long needles characteristic of chymotrypsinogen. Hence the pressure resulted in a partial denaturation rather than the formation of a new zymogen of lower activity after trypsin treatment.

Effect of Pressure on Activation of Chymotrypsinogen by Trypsin

Procedure—The solutions of chymotrypsinogen and trypsin used contained 1.05 and 0.00067 mg. of protein nitrogen per ml., respectively. 2
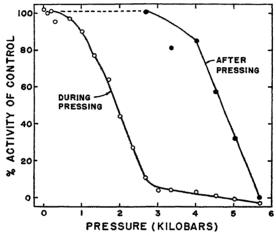
FIG. 3. The effect of pressure on the activation of chymotrypsinogen by trypsin. Pressing time, 60 minutes; pressure as kilobars; concentrations of chymotrypsinogen, 1.05 mg. of protein nitrogen per ml., and of trypsin, 0.00067 mg. of protein nitrogen per ml.; pH, 7.6.

ml. of chymotrypsinogen solution were mixed with 6 ml. of 0.1 M phosphate buffer, pH 7.6. 2 ml. of trypsin solution were then added, the time being noted. One-half was introduced into the pressure chamber as rapidly as possible, the other half was held at 25° as a control. The pressure was raised to the desired level, the time again being noted. After exactly 60 minutes the pressure was released. 1 ml. aliquots of both the pressed sample and the control were added to 4 ml. of N/15 hydrochloric acid, the times again being noted, and aliquots were assayed by the method of Anson (15). In some cases aliquots of the pressed solution which had stood for exactly 60 minutes after release of the pressure were assayed as above. The object here was to study the effect of the 1 hour pressing on the rate of activation after release of the pressure.

The activities for the control sample and for the pressed sample were calculated on a 60 minute basis, the actual elapsed time being around 68 minutes. For the pressed sample, the correction for the time between mixing the solution and applying the pressure was calculated from the activity of the corresponding control. The same method was used for the time after release of the pressure until mixing an aliquot with hydrochloric acid, for pressures up to 2700 bars. Above this pressure, the activation proceeded at a slower rate after release of the pressure than initially in the control, and therefore the correction was calculated from this slower rate.

Results—The results are given in Fig. 3. Within the limit of experimental error, no acceleration in the rate of activation of chymotrypsinogen by trypsin under pressure was found. Up to 1000 bars, the pressure had little or no effect, but above this point the per cent activity of the pressed solution decreased rapidly with increase in pressure until it was nearly 0 at 3000 bars.

DISCUSSION

Pepsin—The results obtained with pepsin differ in several important respects from those obtained with trypsin and chymotrypsin (1). The pH optimum for maximum retention of activity was at 3.5 to 4.0, with sharp decreases above or below this range; no decrease was observed in trypsin or chymotrypsin at low pH values. The maximum per cent retention of activity with pepsin was found at the highest concentrations tested, which was the reverse with trypsin and chymotrypsin. With pepsin, increase in pressure or pressing time invariably resulted in a decrease in the activity; there was no tendency for a part of the activity to be very resistant to inactivation by pressure as in the other two enzymes.

As shown above, the results obtained with pepsin differ in several important respects from those obtained with trypsin and chymotrypsin (1). The following simple mechanism, in which P represents pepsin under ordinary conditions and iP pressure-inactivated pepsin, is in accord with most of the pepsin results.

$$P \xrightarrow{\text{p}} iP$$

However, it can be seen from Table II that three 5 minute pressings produced a considerably greater effect than a single 15 minute pressing. Apparently there is an effect of the application of pressure alone. The following scheme, which is similar to that postulated for trypsin and chymotrypsin, and in which Pa represents reversibly pressure-changed pepsin (folded or aggregated), is more consistent with all of the results.

$$iP \xleftarrow{\quad P \quad} P \underset{\longrightarrow}{\overset{P}{\rightleftharpoons}} Pa$$

In the case of pepsin, however, it is postulated that the reaction to form
Pa would not go so nearly to completion under pressure, as it would in the
case of trypsin or chymotrypsin, and that even under pressure the Pa
would revert to P and be continuously converted to iP. This scheme is
advanced as a tentative hypothesis and not as a final and complete explana-
tion.

The curve (Fig. 1) for the influence of pH on the effect of pressure on
pepsin is similar to the corresponding curves for trypsin and chymotrypsin
in that retention of activity drops sharply with increase above pH 4. Ap-
parently all three enzymes are quite sensitive to hydroxyl ions. Trypsin
and chymotrypsin are most active at about pH 8, and are quite readily in-
activated by pressure at this pH. Pepsin, on the other hand, is most
active at around pH 2, and we find that with increasing acidity below pH
3.5 pepsin becomes less stable under pressure. Trypsin and chymotrypsin
are quite inactive at low pH and are also very little affected by pressure
under these conditions. It is probable (1, 10) that when an enzyme is
active it is partially unfolded, and when in this condition is much more
susceptible to pressure than when it is inactive (but not denatured) and
probably in a more folded or globular form.

Chymotrypsinogen—The effect of pressure on chymotrypsinogen is similar
in most respects to effects on trypsin and chymotrypsin but it is somewhat
more stable under the same conditions. Unlike the two enzymes, chymo-
trypsinogen was little, if any, further affected by repeated applications of
pressure. It appears that under pressure a portion of the chymotrypsino-
gen is converted to a pressure-resistant form $(Xtg)_a$, still convertible to
chymotrypsin, while the remainder is changed to a form not convertible to
chymotrypsin $(Xtg)_d$.

Unlike trypsin and chymotrypsin, chymotrypsinogen was little, if any,
further affected by repeated applications of pressure. It appears that the
pressure-resistant form $(Xtg)_a$ does not readily revert to the original form
on release of the pressure, since in one experiment a 22 hour period between
two pressings yielded results identical with those obtained in similar ex-
periments with a single 5 minute pressing. It does not appear probable
that the high pressure merely converts chymotrypsinogen to another form
which reacts more slowly or less completely with trypsin, since in one ex-
periment in which the pressed solution was fractionated, amorphous mate-
rial of low activity (after trypsin treatment) was obtained, and finally
some crystalline material apparently identical with chymotrypsinogen. It
appears that during the fractionation, which took place over a period of

several weeks, a considerable part of the $(Xtg)_a$ reverted to the original form.

The accompanying scheme is proposed to account for the observations:

$$Xtg \xrightleftharpoons[\text{very slow}]{p} (Xtg)_a$$
$$\downarrow p$$
$$(Xtg)_d$$

where Xtg is the original chymotrypsinogen, $(Xtg)_a$ is a pressure-resistant form still activatible by trypsin, but only very slowly reverting to Xtg, and $(Xtg)_d$ is irreversibly denatured chymotrypsinogen not activated by trypsin.

The conversion of chymotrypsinogen to the active enzyme by trypsin is most probably a hydrolytic process. Since Linderstrøm-Lang and Jacobsen (16) have shown that the hydrolysis of proteins is accompanied by a decrease in volume, it might be expected that pressure would favor this conversion. However, no acceleration by pressure was observed, but rather inhibition. It has previously been suggested (1, 10) that an enzyme must be partially unfolded to be active and that pressure opposes the unfolding. In the present case both the enzyme and the substrate (chymotrypsinogen) may need to be partially unfolded. Such a hypothesis is consistent with the results obtained.

SUMMARY

The effect of high pressures on pepsin and chymotrypsinogen has been investigated, including the influences of pH, concentration, magnitude of pressure, duration of pressing, and multiple pressing. The results with pepsin differed in several important respects from those previously obtained with trypsin and chymotrypsin. The results with chymotrypsinogen were similar to those with trypsin and chymotrypsin in all respects, except that it was not significantly affected by multiple applications of pressure. Possible explanations of these differences are discussed.

Chymotrypsinogen was not activated by pressure. The activation of chymotrypsinogen by trypsin was not accelerated by pressure; it was appreciably inhibited by a pressure of 1000 bars, and almost completely stopped by a pressure of 3000 bars. At this pressure the activation proceeded at almost the same rate after release of the pressure as in the control. At a pressure of 5700 bars, however, no activation occurred after release of the pressure.

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SEPARATION OF CHONDROITIN SULFATE FROM CARTILAGE*

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Isolation of chondroitin sulfate involves extraction of cartilage with 2 per cent sodium hydroxide, followed by procedures to remove protein. That treatment with alkali may cause degradation of chondroitin sulfate has been pointed out by Jorpes (1) and Meyer *et al.* (2). Such treatment also degrades cartilage collagen and dissolves some. This protein is difficult to remove from the mucopolysaccharide. Meyer and Smyth (3) used calcium chloride solution instead of alkali for the initial extraction of chondroitin sulfate. This method avoids possible alkaline degradation of the polysaccharide, but also dissolves collagen and must be followed by lengthy procedures for removal of protein. Blix and Snellman (4) used this calcium chloride extraction method to prepare chondroitin sulfate in a pure and undergraded form and got yields of about 1 per cent of the dry weight of the cartilage.

Procedures are now described for extraction of cartilage under conditions in which little protein is extracted; therefore, purification of the chondroitin sulfate can be simplified.

EXPERIMENTAL

Chondroitin sulfate is determined in cartilage by measurement of the hexosamine content by the procedure of Elson and Morgan (5) as improved by Schloss (6). Since the method of Schloss is not readily accessible, its essential points are given here. A weighed sample containing about 2 mg. of hexosamine is sealed in a 4 inch glass test-tube with 2 ml. of 4.00 M HCl and heated in a bath at 100° for 8 hours. The solution is diluted to 50 ml. with water, shaken with about 0.5 gm. of Darco G-60, and filtered. To 2 ml. of the filtrate in a 25 ml. volumetric flask are added 5.5 ml. of acetylacetone reagent; the mixture is heated in a boiling water bath for exactly 20 minutes, quickly cooled, and about 10 ml. of absolute alcohol are added with mixing; 2.5 ml. of Ehrlich's reagent are added and the volume is made up to 25 ml. with absolute alcohol. After 24 hours, the optical density of the solution is compared at 530 mμ with a standard

curve. The acetylacetone reagent is prepared from 4.76 gm. of anhydrous Na_2CO_3, 0.89 ml. freshly distilled acetylacetone (b.p. range 135–136°), and 7.81 ml. of 1.00 M HCl, and made up to 100 ml. with water. The Ehrlich reagent is made by dissolving 3.2 gm. of recrystallized p-dimethylamino-benzaldehyde in a mixture of absolute alcohol (120 ml.) and concentrated hydrochloric acid (120 ml.).

Fresh cattle tracheae were cleaned of all non-cartilaginous tissue and ground. This material is called fresh wet cartilage. The hexosamine content of six preparations was between 1.26 and 1.49 per cent and averaged 1.36 per cent. A second starting material, stored wet cartilage, was prepared from the fresh wet cartilage (500 gm.) by sprinkling it with thymol (10 gm.) and storing at 0° for 4 to 6 weeks. A third starting material, acetone-dried cartilage, was prepared from fresh wet cartilage by dehydration with several changes of acetone and drying to constant weight in air. For six such preparations, the weight of dried cartilage averaged 33.4 per cent of the weight of the fresh wet cartilage. The hexosamine content of acetone-dried cartilage was between 3.81 and 4.43 per cent and averaged 4.20 per cent. This figure is used in the calculation of yields of chondroitin sulfate. Four methods were compared for extracting the mucopolysaccharide from cartilage.

Method A_1—Acetone-dried cartilage (50 gm.) was suspended in aqueous 30 per cent KCl solution and shaken for 2 days. The liquid was drained off and the residue was again shaken with a liter of fresh KCl solution for 2 days. The residue was worked up separately as described below. To the combined cloudy liquid extracts were added acetic acid (20 ml.) and potassium acetate (20 gm.); the solution was stirred for a half an hour with kaolin (20 gm.), filtered with suction through Filter-Cel, and dialyzed for 1 day against running water. To the clear liquid were added acetic acid (20 ml.), potassium acetate (20 gm.), and kaolin (20 gm.). The mixture was stirred for half an hour and filtered, and the colorless filtrate was evaporated *in vacuo* to about 200 ml. Potassium acetate (10 gm.) was added and the solution was poured into absolute alcohol (1000 ml.), producing a white flocculent precipitate. After several hours, this was centrifuged, washed with absolute alcohol and absolute ether, and dried *in vacuo*. The average yield was 275 mg., containing 16.3 per cent hexosamine or only 2.1 per cent of the chondroitin sulfate present in the cartilage.

For purposes of checking the total yield obtainable, the residual cartilage, left after extraction with potassium chloride solution, was suspended in 2 per cent potassium hydroxide solution (1 liter) at 0° for 2 days without shaking. The liquid was drained and, after acidification with acetic acid, treated exactly as above. The average yield was 6.70 gm., containing 24.6 per cent hexosamine or 78.5 per cent of that present in the cartilage.

Method A_2—This procedure is identical with Method A_1 except that the extracting potassium chloride solution contained, in addition, 1 per cent potassium carbonate. The average yield was 4.95 gm., containing 25.5 per cent hexosamine or 60.0 per cent of that present in the cartilage.

Method B_1—This is identical with Method A_1 except that fresh wet cartilage (150 gm.) is used instead of acetone-dried cartilage (50 gm.). The average yield was 1.05 gm., containing 22.2 per cent hexosamine or 11.1 per cent of that present.

Method B_2—This is identical with Method B_1 except that stored wet cartilage is used instead of fresh wet cartilage. The average yield was 6.65 gm., containing 23.1 per cent hexosamine or 73.1 per cent of that present in cartilage.

All results are averages of not less than six independent experiments. Method A_1 confirms what is already known. Method A_2 shows that with potassium chloride solution containing 1 per cent potassium carbonate 60 per cent of the mucopolysaccharide can be extracted from the cartilage. From fresh wet cartilage, potassium chloride solution extracts only 11 per cent of the chondroitin sulfate, but, after storage of cartilage at ice temperature, it extracts 72 per cent of the mucopolysaccharide. In this extraction no alkali at all is used.

Methods A_2 and B_2 have an advantage in being extraction procedures involving milder conditions than the sodium hydroxide method usually used. Furthermore, less protein is simultaneously extracted. This is shown in Fig. 1 in which the absorption spectra of products made by several methods are compared in the wave-length range 240 to 340 mμ. For comparison, products were made by extraction with 10 per cent calcium chloride and also with 2 per cent sodium hydroxide, followed by the isolation procedure described under Method A_1. The absorption curves of these products are Curves A (CaCl$_2$ extraction) and B (NaOH extraction) of Fig. 1. The product obtained by calcium chloride extraction contains a large amount of protein. Considerable amounts of protein dissolve when collagen is shaken with calcium chloride solution. Potassium chloride solution dissolves little collagen. Chondroitin sulfate extracted from cartilage by Method A_2 gives Curve C. This shows a lower protein content than the products extracted by either sodium hydroxide or calcium chloride.

The methods of separation of chondroitin sulfate described, in which the system is first flooded with potassium and then dialyzed and finally precipitated in the presence of excess potassium acetate, insure that the product will be a neutral potassium salt and not a mixed salt or an acid salt. Because the product contains at this stage relatively little protein, it is easy to purify. For this purpose the product made by Method A_2 or B_2 is dissolved in water (3 gm. in 100 ml.), acidified with acetic acid (2 ml.), and stirred with kaolin (4 gm.) for half an hour. After filtering
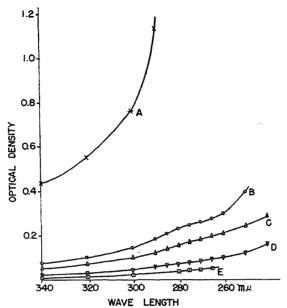
Fig. 1. Relation between optical density and wave-length for potassium chondroitin sulfate preparations at a concentration of 3 mg. per ml. Curve A, extracted from dry cartilage with $CaCl_2$ solution; Curve B, extracted from dry cartilage with NaOH solution; Curve C, extracted from dry cartilage with $KCl-K_2CO_3$ solution (Method A_2) or extracted from wet cartilage with KOH following previous extraction with KCl (Method B_1). Curves D and E, extreme limits of products made as in Curve C and purified by further kaolin treatment.

TABLE I

Experimental and Calculated Values in Per Cent for Some Constituents of Purified Potassium Chondroitin Sulfate

Method	Hexosa-mine	Sulfate	Potassium	Nitrogen	Water
A_2, $KCl-K_2CO_3$ extraction.........	22.57	12.26	11.04	2.18	11.42
	26.36	13.29	11.30	2.08	10.06
	22.43	13.55	10.95	2.07	10.85
B_2, KCl "	23.06	13.16	10.49	1.98	10.22
	23.48	11.80	11.92	2.51	11.48
A_1, KOH "	23.52	12.21	12.58	2.21	10.69
B_1, KOH "	23.45	11.25	10.57	2.01	10.88
Average........................	23.55	12.50	11.27	2.15	10.80
Calculated					
$C_{14}H_{19}NSO_{14}K_2$.................	33.45	17.94	14.58	2.62	0.0
$C_{14}H_{19}NSO_{14}K_2 + 4H_2O$.......	29.49	15.81	12.84	2.31	11.86

through Filter-Cel, the solution is again stirred for half an hour with 4 gm. of kaolin and filtered. Potassium acetate (10 gm.) is added to the filtrate and absolute alcohol (300 ml.) is stirred into the solution. The precipitate is centrifuged, washed with absolute alcohol and absolute ether, and dried *in vacuo*. The yield is 2.1 gm. Such products show absorption curves between Curves D and E of Fig. 1. No treatment has been found which gives a lower absorption curve. The purified product gives no positive protein test with any protein precipitant nor with the biuret reagent. The test for glycogen is negative. When an aqueous solution is shaken for 1 hour with chloroform and amyl alcohol, no trace of emulsion is formed. A summary of analytical data for several preparations is given in Table I.

<div align="center">DISCUSSION</div>

Reports of chondroitin sulfate content of cartilage show a wide range of values; Winter (7) found 41 per cent for pig nasal septum, Miyazaki (8) found 36 to 39 per cent for nasal septum or tracheal cartilage of ox, horse, and pig, Meyer and Smyth (3) found 25 per cent for a dry cartilage of unspecified origin, Hass and Garthwaite (9) found 20 per cent for human infant epiphyseal cartilage, and Partridge (10) found for cattle nasal septum a sulfur content equivalent to 20 per cent. Our value for cattle tracheal cartilage, based on hexosamine, corresponds to 12.6 per cent chondroitin sulfate as the potassium salt. For cattle nasal septa, we have found between 19 and 23 per cent chondroitin sulfate.

Methods for the extraction of chondroitin sulfate have sometimes been especially designed to carry protein into solution. Furth and Bruno (11) first heated cartilage in water so that on subsequent treatment with alkali almost the entire cartilage substance dissolved. The method of Meyer and Smyth (3) originated in the observation that gelatin complexes of chondroitin sulfate were completely soluble in aqueous calcium chloride solution. The methods used in the present work evolved from attempts to find conditions of extraction of mucopolysaccharide that would dissolve the least amount of protein, thus greatly simplifying subsequent purification. Although alkali still must be used, 1 per cent potassium carbonate is sufficient to permit extraction of over half the mucopolysaccharide present. Previous workers have found stronger alkali necessary for extraction of the chondroitin sulfate from cartilage, Jorpes (1) reporting solutions of sodium hydroxide of less than 2 per cent inadequate. Extraction of chondroitin sulfate from stored wet, but not from fresh wet, cartilage, without the use of alkali at all, suggests that the binding of chondroitin sulfate and collagen is other than salt-like and that the linkage may be broken by enzymatic processes.

Analytical data on the purified products, Table I, do not agree with the accepted formula. Chondroitin sulfate prepared by Blix and Snellman (4), probably one of the purest reported, gave a low value for hexosamine, 24 to 25 per cent. Meyer and Smyth (3), though reporting a higher value for hexosamine, 29 per cent, found a low and variable ash. Wolfrom et al. (12) reported higher hexosamine, 31 per cent, and a high value for sodium. Our results show an integral ratio of hexosamine, sulfate, potassium, nitrogen equal to 0.94, 0.93, 2.06, and 1.10, but the absolute values are all too low. This point needs further investigation.

SUMMARY

New procedures are described for the isolation of chondroitin sulfate from beef tracheal cartilage.

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STUDIES ON THE STABILITY OF THE CHOLINE OXIDASE

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The choline oxidase was first described by Bernheim and Bernheim (1)· Later it was shown (2) that choline at pH 6.7 was oxidized to betaine aldehyde which was then only slowly oxidized further, whereas at pH 7.8 the oxidation proceeded rapidly to betaine. This indicated that betaine aldehyde was oxidized by another enzyme system, and Klein and Handler (3) were able to show that its oxidation required DPN[1] but that of choline did not. The choline oxidase is inhibited by fatty acids (4) and its activity is greatly depressed in fatty livers (5). Its importance in the development of fatty livers caused by choline deficiency is shown by the fact that guinea pigs which lack the enzyme cannot be made to develop fatty livers (6) and hamsters which have some enzyme but much less than rats never accumulate as much liver fat on a choline-deficient diet as rats do (7). The enzyme can thus be considered a disadvantage when minimal amounts of choline are present for fat transport and metabolism. On the other hand, the recent work of Dubnoff (8) and Muntz (9) shows that it plays an important rôle in transmethylation reactions, for apparently only betaine aldehyde or betaine can act as a methyl donor. Animals which lack the enzyme can therefore presumably obtain their methyl groups from methionine only. Because of the importance of the enzyme a further study of its properties seems in order. It has previously been shown (1) that it is cyanide-sensitive and that it is readily inhibited by copper and phenylhydrazine (10). Barron and Singer (11) place it in the group of sulfhydryl enzymes. It is very sensitive to diamidines (12). The following is a study of the stability of the enzyme and the effect of cystine and certain metal ions upon it.

EXPERIMENTAL

10 to 12 gm. of rat liver were chopped with scissors, ground in a mortar with 10 to 15 ml. of 0.05 M K-Na-phosphate buffer of pH 6.7, and squeezed through muslin. The suspension was diluted to 50 ml. with water and centrifuged 10 minutes, and the supernatant discarded. 10 ml. more of buffer and 40 ml. more of water were added to the solid which was mixed

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[1] Diphosphopyridine nucleotide.

and centrifuged again. The resulting solid was mixed with 1.0 to 2.0 ml. of buffer and 0.5 ml. used in each Warburg vessel; the total fluid volume was 2.0 ml. Without added substrate the suspension took up little oxygen.

The rate curves thus obtained are complex. On the assumption that the oxidation of choline is a first order reaction, the aldehyde oxidation being disregarded for the moment, log $(a - x)$ was plotted against t. The values chosen for a corresponded to the uptake of 1 atom of oxygen for each molecule of choline. The curves obtained in this way were of considerable diversity, but could be divided into three groups.

In the first group all points fell on a straight line; there is therefore no oxidation of betaine aldehyde in these experiments, and the oxidation of choline is a first order reaction. In the second group, although the points tended to fall on a curve, in many instances this was true only after 30 to 60 minutes or more, the earlier points falling on a straight line. This indicates that after an interval oxygen uptake became faster than expected. In these cases the total oxygen uptake exceeded the amount calculated on the basis of 1 atom of oxygen for each molecule of choline. The increased rate is therefore due to oxidation of the aldehyde, beginning somewhat later. It has been unfortunately impossible to analyze the data for the second reaction, but in general it apparently tends to begin earlier when the velocity constant of the first reaction is greater, $i.e.$ at low substrate concentrations. The reason for the delay is unknown.

In the third group the points fell on a curve convex to the left, and in no case was there an initial linear phase. In these experiments oxidation ceased before 1 atom of oxygen was taken up per molecule of choline, although there was no constant end-point. Inactivation of enzyme was the probable cause of the deviation, and this was shown to be the case, both in the presence and absence of substrate. The effect was twice as great at pH 6.7 as at pH 7.8. Thus, if the enzyme suspension was incubated 45 minutes at 37° before the addition of choline, the rate of oxidation was about half the original at pH 6.7 and about three-fourths at pH 7.8. Because the loss of activity was greatest initially, the assumption was made that the inactivation of enzyme was also a first order reaction, $i.e.$ $dE/dt = cE$, where E is concentration of active enzyme at t minutes and c is a constant characterizing the rate of inactivation. On integration this gives $E = E_0 e^{-ct}$, E_0 being the concentration of active enzyme at zero time. On the additional assumption that the rate of oxidation of choline is proportional to the concentration of active enzyme, the rate equation becomes $dx/dt = -kE(a - x)$. On substituting for E and integrating, this becomes

$$\log \frac{a}{a - x} = \frac{k}{c} E_0(e^{-ct} - 1) \tag{1}$$

Accordingly if log $(a - x)$ is plotted against $(e^{-ct} - 1)$, the points should fall on a straight line, and this was found to be the case (Fig. 1). It was possible to change every curve of the third group into a straight line by choosing appropriate values for c and plotting in this way. The slope, $(k/c)E_0$, for each of these lines was calculated by the method of least squares; its value multiplied by c gave kE_0.

To determine the rate of inactivation of the enzyme in the absence of substrate, the enzyme preparation was placed in the Warburg vessels at zero time and the substrate added at t_1 minutes. Assuming that the en-
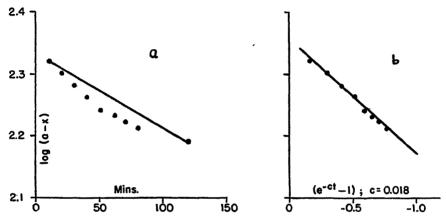
FIG. 1. (a) Log $(a - x)$ plotted against time assuming no inactivation of enzyme. (b) The same plotted against $(e^{-ct} - 1)$ on the assumption that the enzyme is inactivated at a rate given by $c = 0.018$.

zyme concentration has decreased to E_1 at t_1 minutes, equation (1) becomes

$$\log \frac{a}{a - x} = \frac{k}{c_1} E_1 e^{-c_1(t - t_1)} - 1 \qquad (2)$$

c_1 being the constant of the rate of inactivation in the presence of substrate. kE_1 is calculated as before. If inactivation follows the same rule in the absence of substrate, the concentration of active enzyme when the substrate is added will be given by $E_1 = E_0 e^{-c_2 t_1}$ or $\ln kE_1 = kE_0 - c_2 t_1$, c_2 being the inactivation constant in the absence of substrate. If several determinations of kE are made at different times, their logarithms plotted against t should fall on a straight line. This was found to be the case (Fig. 2). The slope of the line, 0.018, is equal to c_2, the constant of inactivation in the absence of substrate.

Because inactivation is a first order reaction, either the enzyme is in itself a very unstable molecule, or inactivation results from a reaction with

some substance present in sufficiently great amount so that its concentration is not significantly altered by any combination with enzyme. Inactivation occurred whether the vessels were shaken or not, which indicates that surface denaturation of the enzyme is not the cause. It also occurred equally well at oxygen tensions of 2 per cent and in the presence of sufficient cyanide to inhibit the oxidation of choline about 30 per cent. The presence of substrate, however, decreased the rate of inactivation; in the experiment cited above, for example, c_1 (presence of choline) varied from 0.004 to 0.010 with different substrate concentrations, while c_2 (absence of choline) was 0.018.
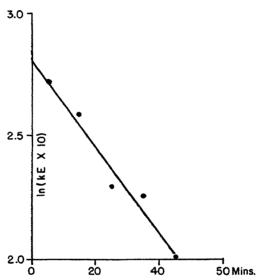
FIG. 2. Inactivation of enzyme in absence of substrate. The time of shaking the enzyme is plotted against $\ln (kE \times 10)$. See the text. The slope of the line, 0.0176, is equal to the constant of inactivation.

Cystine was found to accelerate inactivation very markedly at pH 6.7, less at pH 7.8. The effect of L-cystine in the presence of choline is shown in Fig. 3; in the absence of choline the rate of inactivation was considerably greater. Oxidized glutathione in equimolecular concentrations did not affect the rate of inactivation. It was assumed that cystine oxidized the —SH groups on the enzyme and that it or a similar substance in the preparation accounted for the "spontaneous" inactivation which would therefore be independent of the oxygen pressure. The effect is, however, fairly specific. In the first place, copper ions which are a general catalyst for —SH groups do not catalyze the inactivation of the choline oxidase as

cystine does. 1×10^{-4} M copper sulfate when added with choline to the enzyme caused an 18 per cent inhibition; if added 30 minutes before the choline, the inhibition was 19 per cent; substrate therefore does not protect. The corresponding figures on the same preparation for 0.83×10^{-3} M cystine were 20 and 63 per cent, showing marked protection. Secondly, the succinoxidase which also contains —SH groups is not inactivated by incubation with or without cystine under similar conditions, although it is readily inactivated by copper. The effect of incubation with cystine on the choline and succinoxidase at similar rates of oxidation is shown in Table I. Finally, DL-cystine was as effective in accelerating inactivation as an equimolecular amount of L-cystine. In one experiment the value of

FIG. 3.   FIG. 4.
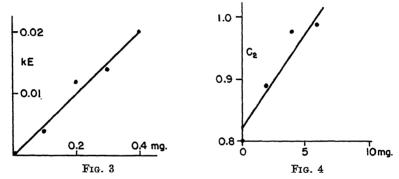
FIG. 3. Inactivation of enzyme by cystine in presence of substrate. The product kE is plotted against the amount of cystine per 2 ml. It is assumed that the change in kE represents a change in E, the concentration of enzyme, only.

FIG. 4. Inactivation of enzyme by semicarbazide in the absence of substrate. The velocity constant (c_2) of inactivation is plotted against the amount of semicarbazide added to the 2 ml. of fluid in the reaction vessel.

kE_1 for enzyme shaken 30 minutes in the presence of 0.4 mg. of L-cystine was 0.0051; with the same amount of DL-cystine it was 0.0055. In the absence of cystine the destruction was less, kE_1 being 0.008. Both forms of cystine are as effective in crude liver suspensions as in the partially purified preparation.

In an attempt to block the second oxidation, that of the aldehyde, semicarbazide was added. This also had the effect of increasing the rate of inactivation of the enzyme both in the presence and the absence of substrate (Fig. 4); the rate of inactivation was again greater in the absence of choline (c constants 0.032 and 0.0012 per mg. of semicarbazide respectively).

It seemed probable that nickel, cobalt, and iron salts would also react with —SH groups, partly as catalysts of their oxidation, but also by form-

ing complexes with the enzyme. In order to investigate this, the metals as sulfates were dissolved in water and added to the liver suspension in phosphate buffer. The effect on the rate of inactivation is shown by an experiment in which the constant in the presence of choline was found to be 0.001; on the addition of 22 γ of cobalt it rose to 0.012. Further addition of cobalt had little effect on this value. In the second place the metallic ions inhibit the enzyme. To determine whether this inhibition is competitive or not, the Michaelis constant was estimated by determining the values for kE_0 (equation (1)) for three or four different concentrations of choline. Five such experiments were carried out and the values of K_m on successive pooling of data were 0.0022, 0.0012, 0.0015, 0.0016, and

TABLE I

Effect of 30 Minute Incubation with and without 0.83 × 10⁻³ м Cystine at 37°
on Activity of Choline and Succinoxidases

0.5 ml. of liver suspension was used for the choline oxidase, 0.1 ml. for the succinoxidase. 2.0 mg. of each substrate were used. The figures are in c.mm. of O_2 uptake. The appropriate control uptakes have been subtracted.

Min.	No incubation				30 min. incubation			
	Choline	Choline + cystine	Succinate	Succinate + cystine	Choline	Choline + cystine	Succinate	Succinate + cystine
10	28	27	16	14	18	1	14	16
20	51	45	35	31	33	3	36	31
30	68	58	55	49	47	8	54	46
40	84	67	74	72	57	9	74	62
50	94	71	92	85	67	11	89	78
60	103	76	111	93				
70	110	80	124	110				
80	118	81	136	124				

0.0017, with 13 degrees of freedom when values are expressed in moles, liters, and minutes. The F test showed that these differences were not significant. On the addition of 22 γ of nickel to the reaction mixture, successive pooling gave 0.0096, 0.0088, and 0.0100 for the value of the slope, that is for $K_m(1 + (i/K_i))$, where i refers to the molar concentration of inhibitor and K_i is the inhibitor-enzyme dissociation constant. There were 7 degrees of freedom and the F test showed that the variation in different determinations was not significant. The difference between this figure and the value for K_m is sufficiently great to be beyond the range of experimental error and indicates that this is a case of competitive inhibition. The number of atoms of metal combining with 1 molecule (or active center) of enzyme is determined by plotting the reciprocal of the

initial velocity against the concentration of inhibitor. In every experiment with all three metals a straight line was obtained when the first power of the inhibitor concentration was used, which indicates that 1 atom of the inhibitor combines with 1 unit of enzyme. The value for K_i, from the last figure of each series, was found to be 0.38×10^{-4}. On addition of 42 γ of cobalt successive poolings gave 0.0052, 0.0066, 0.0076, and 0.0074 for the value of the slope. There were 11 degrees of freedom and the F test was not significant. The straight line obtained on plotting the reciprocal of the initial velocity against the first power of the inhibitor concentration is shown in Fig. 5. From these figures K_i was found to be
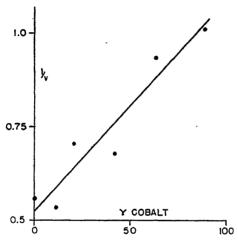
FIG. 5. Effect of increasing concentrations of cobalt sulfate. The reciprocal of the velocity is plotted against the square of the concentration of cobalt sulfate (in micrograms per 2 ml.).

1.1×10^{-4}. On the addition of 37 γ of iron, the successive pooled values of the slope were 0.014, 0.017, and 0.017 with 9 degrees of freedom. Again no significant variation was found by the F test, and the relationship between $1/v$ and the inhibitor concentration was linear. K_i was found to be 0.37×10^{-4}.

These salts are thus effective competitive inhibitors of the choline oxidase, but under comparable conditions have no effect on the succinoxidase, even when the suspension is diluted so that succinic acid and choline are oxidized at the same rates. Other enzymes in the washed suspension, such as L-proline oxidase, amine oxidase, and D-amino acid oxidase, are also unaffected by this concentration of the metals. Manganese in 4 times the concentration does not inhibit the choline oxidase. The af-

finity of the metallic cations for the active groups of the liver protein is high enough, as shown by the dissociation constant, so that insoluble phosphates are not formed to an appreciable extent. This is confirmed by the fact that, if the phosphate concentration is lowered, the inhibition of the enzyme is not altered. Additional evidence is summarized in Fig. 6. When 8-hydroxyquinoline is added before the nickel, no inhibition occurs. If it is added 30 minutes after the nickel, the inhibition is only very slowly decreased, which indicates a very small rate of dissociation of the nickel enzyme complex.
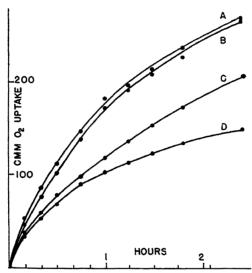
FIG. 6. The effect of 8-hydroxyquinoline added before and 30 minutes after 0.1 mg. of NiSO$_4$ on the oxidation of choline. Curve A, control, with 0.5 mg. of 8-hydroxyquinoline; Curve B, 8-hydroxyquinoline added before the nickel; Curve C, 8-hydroxyquinoline added 30 minutes after the nickel; Curve D, nickel alone.

SUMMARY

1. Choline oxidase partially purified rapidly loses its activity. The rate of loss is that of a first order reaction and equations are derived to describe the rate of reaction as modified by this. The inactivation is more rapid at pH 6.7 than at 7.8.

2. Cystine and semicarbazide increase the rate of inactivation both in the presence and absence of choline.

3. Nickel, cobalt, and iron salts not only increase the rate of inactivation, but also combine reversibly with a group on the enzyme to inhibit it. Manganese salts have no effect in comparable concentrations.

4. The specificity of these effects is described.

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SPECTROPHOTOMETRIC ESTIMATION OF PYRIDINE NUCLEOTIDES IN ANIMAL TISSUES*

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Investigations in progress in our laboratory require a rapid and accurate method for the estimation of pyridine nucleotides (PN) in animal tissues. Existing methods for the estimation of PN include the determination of the extinction coefficient at 340 mμ of solutions which have been reduced by hydrosulfite (1), the growth rate of *Hemophilus influenzae* or *Hemophilus parainfluenzae*, which have a growth requirement for PN (2), and the enzymatic activity of enzyme systems which require diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) as coenzymes (3, 4).

The first method is the most accurate and reproducible and is routinely employed in measuring the concentration of standard solutions of DPN or TPN. This method requires that the solution to be analyzed be free from turbidity or impurities which absorb strongly at 340 mμ, and therefore cannot be used directly on tissue extracts. The second method has the usual disadvantages of a microbiological assay which preclude a rapid, accurate measurement (5). The third method has the disadvantages which accompany enzymatic assays; namely, difficulty in reproducible preparation of enzymes, necessity for adjusting conditions so that the over-all reaction rate is limited by the concentration of the material being measured, and the need for running standard curves with each assay. In view of the rapidity and accuracy possessed by spectrophotometric analyses, the following study was made to develop a quantitative spectrophotometric method which can be applied directly to tissue extracts.

Papers have recently appeared dealing with the isolation of DPN and TPN from biological materials in which adsorption of the nucleotides on activated charcoal was employed in the isolation procedure (6, 7). This suggested the possibility of developing a micromethod for the adsorption on activated charcoal of PN from tissue extracts, which would permit sep-

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aration of the PN from other cellular components and simultaneously concentrate the PN.

To test the utility of charcoal as an adsorbent in a microanalytical method, the following experiment was performed. 0.10 gm. of activated charcoal was placed between glass wool plugs in a Hennessy tube.[1] A solution containing a known amount of DPN at pH 2.5 was added to the column and permitted to flow through the charcoal at a rate up to 30 drops per minute. The solution passing from the column was collected, analyzed spectrophotometrically, and found to contain no DPN. To the column was added 10 per cent pyridine as an eluant, which was allowed to flow through at approximately the same rate. The eluant was collected
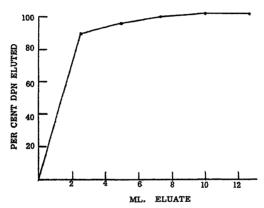
Fig. 1. Elution of adsorbed DPN from Nuchar C by 10 per cent pyridine

in several fractions and analyzed for DPN. Typical results are shown in Fig. 1. When the adsorbed material was washed with as much as 30 ml. of 2 per cent trichloroacetic acid (TCA), complete recovery still resulted. These experiments indicated that DPN could be adsorbed and eluted quantitatively on such a column, and that the adsorbed material could be extensively washed without loss.   Two similar experiments with TPN gave a mean recovery of 86 per cent.

Rat liver was employed in studies on the application of this method to tissue analysis because of the existence of previous estimates of the PN content of this tissue. The adsorption procedure was found to lend itself readily to tissue analysis; however, great variation in the PN content of tissue extracts was observed, depending on the method of preparing the

[1] Wilkens-Anderson Company, Chicago, Illinois, catalogue No. 7105-CB.

extract. In all cases discussed below, the livers were removed from stunned adult male rats, then frozen in solid carbon dioxide-acetone mixture, and weighed while frozen.

Abnormally low amounts of PN were found when the tissue extract was prepared in the following manner. The weighed frozen tissue was added to 10 ml. of water which had been brought to 100° in a boiling water bath, heated for 5 minutes, homogenized in a Potter-Elvehjem homogenizer, and replaced in the boiling water bath for 1 minute. After recording the volume, the homogenate was centrifuged, aliquots of the supernatant were acidified to pH 2.5, adsorbed on the charcoal column, washed with 2 per cent TCA, and eluted with 10 per cent pyridine. The eluate was spectrophotometrically analyzed for PN. Four rat livers were analyzed in this manner and their respective PN contents were 202, 150, 180, and 127 γ of PN per gm. of liver, wet weight. The recoveries of DPN added to aliquots of supernatant immediately prior to adsorption were 94 and 102 per cent, whereas recoveries of DPN added during the boiling period were 75 and 81 per cent. These low values of PN content of rat liver in conjunction with the destruction of DPN added during the boiling period suggested that PN was destroyed by the high temperature during this extraction procedure.

When, without boiling, the frozen liver was homogenized in 2 per cent TCA, subsequent analysis indicated a several fold increase in the PN of the rat livers (2nd column, Table I). In view of the known lability of reduced PN to acid (8) and the probable simultaneous existence *in vivo* of both oxidized and reduced PN, it seemed reasonable to believe that some reduced PN (PNH_2) had been destroyed by the homogenization in acid. To obtain PN values which represent the sum of the oxidized and reduced forms of PN, homogenization in TCA in the presence of an oxidizing agent was necessary. The oxidant must rapidly convert the reduced acid-labile form to the oxidized acid-stable form. Potassium ferricyanide, when used as oxidant (2), yielded erratic recoveries, since it was adsorbed by the charcoal, appeared in the eluate, and interfered in the spectrophotometric analysis of the eluate. Homogenization in a solution which was 5 per cent with respect to H_2O_2 and 2 per cent with respect to TCA yielded PN values considerably above those obtained in the absence of peroxide (Table I). The difference in PN values in the presence and absence of peroxide represents the PNH_2. Since both DPN and TPN are adsorbed on charcoal under these conditions and absorb equally at 340 mμ, this method does not distinguish between DPN and TPN but rather measures their sum.

The absorption curve of a hydrosulfite-reduced aliquot of an eluate of rat liver is presented in Fig. 2. Comparison with the absorption curve of

PNH$_2$ as reported by Warburg in 1936 (9, 10) indicates the similarity of the two curves and the probable absence of contaminants in the eluate.

TABLE I

*Pyridine Nucleotide Content per Gm. of Fresh Liver with Recovery Values**

Rat No.	PN + PNH$_2$ (+H$_2$O$_2$)	PN (−H$_2$O$_2$)	PNH$_2$ (calculated)	Per cent recovery of added DPN
	γ	γ	γ	
1	878	688	190	
2	835	668	167	
3	755	591	164	
4	743	528	215	93
5	891	655	236	85
6	906	655	251	99
7	845			99
8	806			97
9	790			85
10	770			97
11	842			95
Mean.....	824	631	204	95

* Optical density of 0.840 per 100 γ of PNH$_2$ per ml. was used in calculating the amount of PN (11, 12).
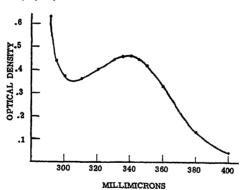
FIG. 2. Absorption curve of hydrosulfite-reduced eluate from rat liver

Procedure

The liver was removed from a freshly killed animal and dropped into a dry ice-acetone mixture. Approximately 2 gm. of the frozen tissue were rapidly and accurately weighed, then homogenized in a 25 mm. diameter Potter-Elvehjem homogenizer for 2 minutes. Homogenization was either in 12 ml. of 2 per cent TCA or 10 ml. of TCA plus 2 ml. of 30 per cent

H_2O_2, depending on whether PN or PN plus PNH_2 was to be determined. The homogenates were permitted to stand at room temperature for at least 5 minutes to complete the extraction of PN; this was followed by centrifugation at 3000 r.p.m. for 5 minutes. Aliquots of the supernatants were added to the charcoal columns.

Nuchar C was the charcoal adsorbent employed.[2] To obtain charcoal particles of a size which would permit a rapid rate of flow through the column, yet possess sufficient surface area for adsorption, the following method was employed. Unground Nuchar C was ground in a Wiley mill through a medium sieve; then the particles which passed through a No. 60 sieve were discarded. The remaining charcoal particles were of proper size and were acid-washed (7). 0.2 gm. of Nuchar C prepared in this manner has an adsorptive capacity for approximately 2 mg. of PN.

The adsorption columns were prepared in the following manner. 0.2 gm. of dry acid-washed Nuchar C was added to the Hennessy tube over a thin glass wool plug and covered by another glass wool plug. The latter was necessary to prevent the charcoal from floating. The Hennessy tubes were fitted with rubber stoppers to permit insertion into suction flasks, since application of suction accelerated the rate of flow through the column. Immediately prior to use of the column approximately 15 ml. of 2 per cent TCA were rapidly drawn through the column with suction. When the level of TCA approached the top of the charcoal, suction was removed and the solution to be adsorbed was added to the column. The supernatant was permitted to flow through the column at a rate of about 25 drops per minute. Frequently, when H_2O_2 was employed, bubbles of oxygen formed in the column and diminished the rate of flow. Slight vacuum applied to the suction flask readily renewed the proper rate of flow. Following complete passage of the supernatant through the column, 15 ml. of 2 per cent TCA were added to remove any non-adsorbed impurities. Half of this was rapidly drawn through the column in a steady stream and the remaining half was allowed to drip rapidly through under gravity. Then 11 ml. of 10 per cent pyridine were added, allowed to flow through under gravity, and the eluate collected in a graduated container. The volume of eluate was recorded and aliquots were removed for spectrophotometric analysis of PN.

A modification of the spectrophotometric method of Gutcho and Stewart (11) was employed. To 5 ml. of eluate in each of two test-tubes were added 1 ml. of 0.5 M $NaHCO_3$ and 1.0 and 0.4 ml. of water, respectively. To the latter tube was added, as reducing reagent, 0.6 ml. of a freshly prepared solution of 450 mg. of $Na_2S_2O_4$ in 25 ml. of 0.5 M $NaHCO_3$, and all the tubes were immediately placed in a boiling water bath for 90 seconds.

[2] Obtained from the Industrial Chemical Sales, New York.

They were then cooled in an ice bath and kept there through the following procedure. 1.0 ml. of 0.5 M Na_2CO_3 was added to all the tubes and oxygen was bubbled through for 2.5 minutes. We have found it convenient to employ a gas manifold with eight outlets to permit simultaneous gassing of eight tubes. The solutions were read in a Beckman model DU quartz spectrophotometer at 340 mμ against distilled water. The difference in optical density between the oxidized and reduced samples was due to PNH_2. From the fact that, in a 1 cm. cell, 100 γ of PNH_2 per ml. have an optical density of 0.840 (11, 12) and from knowledge of the volume of eluate analyzed, total volume of eluate, volume of supernatant adsorbed, total volume of homogenate, and weight of tissue, one can readily calculate the PN content of the tissue.

This method has been employed with livers of rats fed a common stock ration. Recoveries were made on known additions of DPN added both prior to homogenization and prior to adsorption. These results are presented in Table I.

DISCUSSION

The method of extraction of the PN from tissues greatly influences the amount found. It is this factor, which has not received sufficient attention in the past, which probably accounts for existing confusion regarding the PN content of rat liver. Dann and Handler (13) found a mean of 175 γ of nicotinic acid per gm. of fresh liver. This is equivalent to 944 γ of DPN; yet they found the actual PN content to be only 396 γ per gm. of fresh liver (14). By using the techniques described in the present report, a mean of 824 γ of PN per gm. of liver has been found. The higher value is probably due to an improved method of extraction and to greater accuracy in measuring the extracted PN.

We have employed this method in other experimental work[3] and duplicate samples of the same liver have an average difference of 2.3 per cent.

SUMMARY
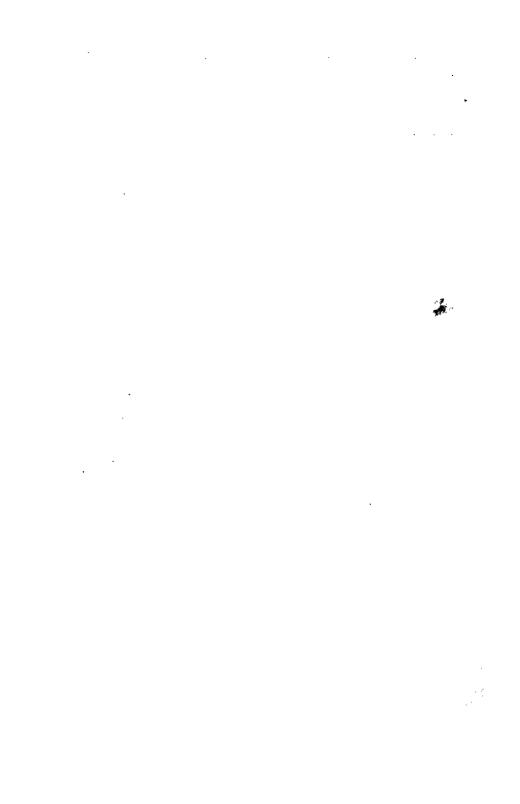
A method is described for the rapid quantitative estimation of the pyridine nucleotide content of animal tissues. The method includes adsorption of pyridine nucleotides from tissue extracts on activated charcoal and the spectrophotometric analysis of the eluate. Analysis of rat liver indicates an average of 824 γ of pyridine nucleotides per gm. of fresh liver when 0.840 (11, 12) is used as the extinction coefficient for 100 γ of PNH_2 per ml.

[3] Unpublished data, Williams, J. N., Jr., Feigelson, P., and Elvehjem, C. A.

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ELECTRON MICROSCOPY OF CRYSTALLINE CATALASE

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EXPERIMENTAL

Catalase was prepared from fresh calf liver and crystallized by the addition of saturated ammonium sulfate according to the method of Sumner and Dounce (1). The suspension of microscopic needles so obtained was dark green in color and showed strong streaming birefringence when viewed between crossed polaroid sheets. The first specimens prepared for electron microscopy were washed with twice distilled water, dried on a collodion film support, shadow-cast, and coated at normal incidence with a thin film of silicon monoxide, according to the method originally used for edestin crystals (2, 3). The protein was finally removed with 0.3 N acetic acid. Electron micrographs of crystal surfaces prepared in this way consisted for the most part of disordered particles, but in small areas rectangular nets were visible. In subsequent preparations, the washed crystals were rinsed briefly with 0.1 per cent phosphotungstic acid in phthalate buffer at pH 5.5 and finally washed again with water. After this treatment, the crystal surfaces were mostly free of disordered material and showed rectangular nets clearly visible over large areas, as illustrated in Fig. 1. It is not clear whether the action of the phosphotungstic acid is such as to stabilize the structure or whether it serves only to remove disordered surface deposits. The crystal fragments are always very thin in a direction normal to the supporting film and are probably denatured.

The micrograph reproduced in Fig. 1 shows a crystal surface on which there is a monomolecular step. Such steps occur frequently, although usually they are long narrow rectangles with their long axis in the direction of the long axis of the fragment, which is designated as the c axis. The step in Fig. 1 shows a tendency to split in this direction. There is an alternation in the intensity of rows of particles aligned in the a direction, resulting in a fundamental c axis period, c_o, consisting of double rows, as shown at higher magnification in Fig. 2. The double nature of c_o is most apparent to the eye when Fig. 1 is viewed in the a direction and at an angle to the plane of the picture. It is not particularly apparent in Fig. 2 because for this surface the direction of the shadows is almost parallel to the a axis, whereas in Fig. 1 it is parallel to the c axis. The double nature of the c axis period is always obvious under proper visual conditions

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in original plates or prints, but the difference between alternate rows is sometimes slight. Particles of higher scattering power in micrographs, such as Fig. 1, are designated A and those in the intervening rows B. The lateral period is designated a_o.

The spacings c_o and a_o may be measured with good accuracy from enlarged prints by measuring the distance occupied by 50 to 100 spacings. Values for c_o varied from 157 to 161 A, with the average at 159 A. Measurements of a_o varied from 61.6 to 65.0 A, with an average of 64 A. The
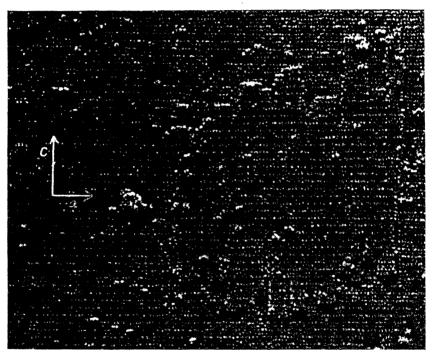
FIG. 1. Surface of a crystal from beef liver catalase showing a monomolecular step. Shadow-cast with platinum. 100,000 ×.

absolute accuracy of such measurements is limited by the errors in calibration, which are judged to be within ±2 per cent. The internal consistency is probably better.

Information concerning the structure perpendicular to the ac plane cannot be obtained with high accuracy because crystals have not yet been observed on edge. Conclusions can, however, be reached concerning this aspect of the structure through a study of monomolecular steps similar to those appearing in Fig. 1. The nets on such steps appear to be, in most instances, in good register with the structure on the lower surrounding

surface. In some micrographs there may be a slight lateral displacement of rows at different levels, but this is usually irregular and has been attributed to distortion due to drying and manipulation. As a result of such observations, it is concluded that the third, or b axis, is approximately perpendicular to the ac plane and that successive layers are in register, as indicated in the diagrammatic representation in Fig. 3. The period b_o was determined by measuring the lengths of shadows at the edge of steps in specimens which had been shadowed with uranium at a shadow to height ratio of 0.33. (Fig. 1 is not suitable for such measurements.) Determinations of the height of steps varied from 57 to 71 A. The final average for b_o was 64 A, which happens to coincide numerically with a_o,
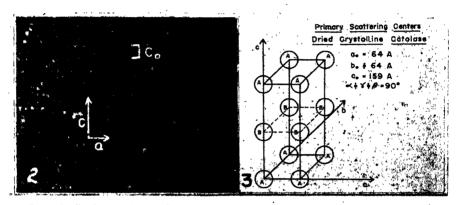
Fig. 2. Surface of catalase crystal showing rectangular molecular net. 200,000 ×.

Fig. 3. Diagrammatic representation of the repeating unit in crystalline catalase. The centers marked A and B are considered to be molecules which differ only in orientation or position. For example, the difference could be due to a displacement of type B with respect to the ac plane.

but this is undoubtedly fortuitous because the uncertainty in b_o might be as large as ±10 per cent.

As shown in Fig. 3, the analysis leads to an approximately orthorhombic cell 64 × 64 × 159 A with two principal scattering centers per cell. In order to calculate the molecular weight, it is necessary to assume an average density for the dry crystal and reach a conclusion regarding the number of molecules in the cell. Assumed densities of 1.30 and 1.35 yield for the equivalent molecular weights of the cell the figures 500,000 and 520,000, respectively, which are greater than published values for the molecular weight of catalase by a factor of almost exactly 2. Satisfactory agreement is obtained if each particle, whether A or B, is considered to represent a single molecule so that there are 2 molecules per cell, as

shown in Fig. 2. The molecular weight from electron microscopy then becomes 250,000 to 260,000, according to whether the assumed density is 1.30 or 1.35. The results will, of course, be subject to revision if better values for the dry crystal density become available. The difference in appearance of alternate molecules A and B presumably reflects a systematic difference in orientation or position. In view of the possible errors in b_o and the uncertainty in the crystal density, these figures are in acceptable agreement with the value of 225,000 given by Sumner, Dounce, and Frampton (4). The agreement is better, however, with the earlier value of 248,000 given by Sumner and Gralén (5) and quoted in a recent publication by Sumner and Somers (6).
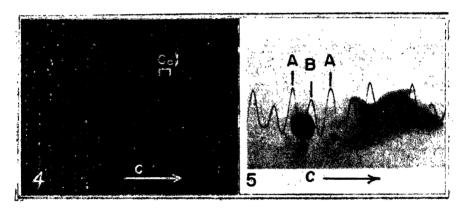
FIG. 4. Micrograph of crystalline catalase similar to those in Figs. 1 and 2, but recorded closer to the true focus in order to show the existence of smaller subunits. 200,000 ×.

FIG. 5. Microphotometer tracing from the micrograph of Fig. 4, demonstrating the period and subperiod along the c direction. Slit dimensions, 14 × 450 A.

In making electron micrographs of this and other proteins, it has been the practice to record each field in a series of five exposures at slightly different focal settings. The micrographs reproduced in Figs. 1 and 2, for example, are from such a series and are appreciably out of focus on the long focal length side. Although the scattering pattern is an approximation to the actual structure, the apparent particles represent the combined scattering effects of smaller units which are resolvable in images recorded closer to the true focal setting. A portion of a micrograph from the same area as that shown in Fig. 1, but at a more accurate focal setting, is reproduced in Fig. 4, wherein smaller particles are readily resolved. Since the order is poor in the smaller dimensions, the pattern which appears in Fig. 2 tends to be lost to the eye. The c_o period is fairly evident, but other

features are vague, including the subperiod in the c direction. That the essential pattern is still present may be demonstrated by running a microphotometer tracing along the c axis, as shown in Fig. 5, which is a portion of a tracing from the micrograph in Fig. 4 recorded with a slit corresponding to 14 × 450 A. There is sufficient integration of the poorly ordered fine structure along the length of the slit to reproduce the half period and to show clearly the alternating intensity of adjacent rows of molecules. The catalase molecule appears to consist of a cluster of smaller units, which have particle weights estimated at 25,000 ± 10,000 as seen in Fig. 4.

An observation on these and other crystal surfaces, which is possibly of some interest, is the appearance of the molecular patterns on crystals which are obviously degenerating. Most often, such surfaces take on a fibrous appearance owing to the coalescence of molecules along principal crystallographic directions. Catalase molecules tend to join along the c direction, while edestin molecules tend to join to form fibrils parallel to the edge of the triangular crystals previously described.

DISCUSSION

Although it has been concluded that the scattering centers shown in Fig. 3 locate the centers of single catalase molecules, the exact nature of the difference between types A and B has not been established. Presumably the differences observed in electron micrographs are due to differences in orientation or to slight displacements from the positions indicated in Fig. 3. If, however, there is a real difference in structure between alternate centers, the length of the molecule would be 159 A instead of one-half this figure. In this event, one or both of the other dimensions would have to be decreased accordingly in order to maintain an acceptable molecular weight. The crystal symmetry cannot be determined with the precision expected of x-ray analysis, but the structure depicted in Fig. 3 should represent a reasonable first approximation to the unit cell. The true cell might, of course, be a multiple of this, owing to variations in orientation or position not perceptible in electron micrographs. Unfortunately, no x-ray diffraction data on crystalline catalase have appeared in the literature for comparison.

The conclusion that the catalase molecule resembles a cluster of much smaller, apparently globular units, is not an isolated instance. Fibrinogen filaments have been observed (7, 8) having dimensions in approximate agreement with molecular dimensions as determined by other methods, but, with adequate resolution, such filaments appear as strings of approximately spherical particles which, individually, are too small to fit molecular data, such as those from flow birefringence (9). A similar aspect was observed in the course of the study of edestin crystals. The

micrographs described in those reports (2, 3) were chosen from focal series in order to show the first order pattern to advantage, but at closer focal settings the molecules are resolved into smaller units which are poorly ordered. It is of interest to note in this case that the x-ray pattern of crystals, even before drying, showed several well defined low order diffractions, while the high order diffractions were weak and many expected diffractions were missing. Unpublished micrographs of several other proteins also give the impression that they are composed of clusters of units smaller than the expected dimensions of the molecule. This circumstance introduces a difficulty in the analysis by electron microscopy, for unless good order can be maintained among such intramolecular components, it is difficult or impossible to locate molecular boundaries. In fact, the major problem encountered in these investigations is that of preserving, through preparative procedures such as washing and drying, the regular structure which presumably exists in protein crystals.

SUMMARY

1. Electron micrographs showing molecular nets at the surface of crystallized beef liver catalase are described. The analysis indicates that the structure is approximately orthorhombic with a unit cell 64 $\times$ 64 $\times$ 159 A and 2 molecules per cell.

2. The volume occupied by 1 molecule is approximately 64 $\times$ 64 $\times$ 80 A.

3. With an assumed density of 1.30 for the dry crystals, the molecular weight is calculated to be 250,000, in satisfactory agreement with data from other methods.

4. Catalase molecules do not appear as sharply bounded particles but rather as clusters of smaller subunits having estimated particle weights in the range 25,000 $\pm$ 10,000.

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THE METABOLISM OF PHENYLALANINE AND TYROSINE IN MUTANT STRAINS OF ESCHERICHIA COLI*

By SOFIA SIMMONDS

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Previous experiments (1) with two biochemical mutants of *Escherichia coli* (strain K-12) which require for growth an exogenous source of biotin and phenylalanine (strain 58-278) and biotin and tyrosine (strain 58-5030) indicated that the amino acid requirement of each mutant is completely specific and that these mutants do not synthesize tyrosine from phenylalanine.

In the present communication, data are presented to show that the *phenylalanineless* strain elaborates a growth factor for the *tyrosineless* strain. Thus, a cell-free filtrate prepared from a culture of the *phenylalanineless* mutant in minimal medium (2) supplemented with biotin and L-phenylalanine shows growth-promoting action for the *tyrosineless* mutant.[1] A culture filtrate from strain 58, a *biotinless* mutant from which strains 58-278 and 58-5030 have been derived, contains only traces of the growth factor for the *tyrosineless* strain.

With the aid of isotopically labeled compounds, it has been possible to demonstrate that culture filtrates from the *phenylalanineless* mutant contain considerable amounts of tyrosine. The organism was grown in minimal medium containing $N^{15}H_4^+$ (23.115 atom per cent excess N^{15}), biotin, and sufficient L-phenylalanine (2 mg.) to permit only half maximal growth in 24 hours. Microbiological assay of the cell-free filtrate with the *tyrosineless* strain as the test organism indicated the presence of 3.76 ± 0.8 mg. of tyrosine. Non-isotopic L-tyrosine (153 mg.) was added to the filtrate as a carrier, and the tyrosine was reisolated. It contained 0.313 atom per cent excess N^{15} (Table I). On the assumption that all of the nitrogen of the bacterial tyrosine had been derived from the ammonia in the medium, it may be calculated that the culture filtrate contained 2.09 mg. of isotopic tyrosine. The discrepancy between this value and 3.8 mg. obtained by the biological assay may be ascribed either to the inaccuracy of the latter method or to the presence, in the culture filtrate, of material other than tyrosine which had growth-promoting activity for the *tyrosineless* mutant.

[1] Preliminary experiments have shown that filtrates from the *phenylalanineless* strain also show tyrosine activity for *Lactobacillus arabinosus*.

To the unwashed bacteria were added 149 mg. of L-tyrosine, and the mixture was hydrolyzed in 6 N sulfuric acid. The tyrosine isolated from the hydrolysate contained 0.171 atom per cent excess N^{15}, which corresponds to 1.11 mg. of bacterial tyrosine. The total quantity of isotopic tyrosine produced by cells grown in the presence of 2 mg. of L-phenylalanine was 3.2 mg., and, therefore, the carbon skeleton of all of the tyrosine produced by the bacteria could not have had its origin in the exogenous phenylalanine. Thus, the mutant must have synthesized tyrosine from the glucose-carbon and ammonia nitrogen supplied in the medium, and a large portion of this tyrosine was elaborated into the medium.

Davis (3) has observed that a *phenylalanineless* mutant derived from *Escherichia coli* strain W (ATCC 9635) also produces material, believed to be tyrosine, which serves as a growth factor for a *tyrosineless* mutant derived from the same parent strain.

TABLE I

Analyses of Isolated Tyrosine

The isotopic ammonia in the medium contained 23.115 atom per cent excess N^{15}.

Sample	N calculated	Tyrosine from filtrate		Tyrosine from cells	
		N found	N^{15}	N found	N^{15}
	per cent	*per cent*	*atom per cent excess*	*per cent*	*atom per cent excess*
2nd recrystallization.................	7.74	7.66	0.311	7.74	0.191
3rd "	7.74	7.73	0.313	7.80	0.171

As reported earlier (4), when the growth of strain 58-278 is studied as a function of time as well as of the initial concentration of phenylalanine in the medium, a series of growth curves is obtained in which the maximum of each curve, *i.e.* the maximal optical density of the bacterial culture, depends upon the initial concentration of amino acid in the medium. It has now been noted that, if the culture tubes in which suboptimal amounts of L-phenylalanine originally had been present are allowed to remain in the incubator for 10 or more hours after these maxima have been attained, there occurs a second period of rapid growth. Fig. 1 shows the diphasic growth curves which are obtained when Evelyn colorimeter tubes, containing 10 ml. of medium and increasing amounts of L-phenylalanine, were inoculated with cells from a stock culture of strain 58-278 and incubated at 30° on a shaker. It will be seen that the length of time required for the initiation of the second growth phase decreases with increasing initial concentrations of phenylalanine. The final maxima of the diphasic curves, however, are approximately the same as that of the monophasic curves ob-

tained in the presence of high concentrations of phenylalanine. Under the conditions of these experiments, no visible growth is observed in the absence of phenylalanine.

Essentially similar diphasic curves are obtained when the initial pH of the medium is 6.4, 7.0, or 7.4, when the temperature during the incubation period is 25°, 30°, or 35°, and when the culture tubes are incubated in a stationary position rather than on a shaker. Such variations in the conditions under which the tests are carried out alter the length of the initial lag period, the length of the lag between the primary and secondary growth phases, and the maxima of the curves.
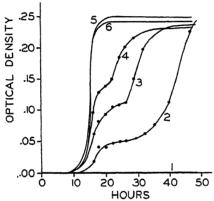
FIG. 1. Diphasic growth curves of strain 58-278 grown in the presence of increasing concentrations of L-phenylalanine. The initial concentration of test compound, in mM per liter: Curve 1, 0; Curve 2, 0.0061; Curve 3, 0.0121; Curve 4, 0.0182; Curve 5, 0.061; Curve 6, 0.182. Density measurements were made at intervals of 2 to 3 hours, but only those points indicative of the diphasic nature of the curves have been shown in the figure.

It seemed likely that the secondary growth of the *phenylalanineless* mutant might be the result of a spontaneous formation of cells that no longer required an exogenous source of phenylalanine for growth. A similar phenomenon has been studied in detail by Ryan and Schneider (5) for a histidine-requiring mutant of *Escherichia coli*. In the case of the phenylalanine-requiring mutant this possibility was tested (a) by sterile transfer of 0.1 ml. aliquots of cultures in which the second growth phase had reached its maximum to liquid minimal medium supplemented only with biotin; (b) by plating aliquots of such cultures on minimal medium solidified with repurified agar (Fisher Scientific Company);[2] and (c) by inoculation of ali-

[2] An apparent loss of the phenylalanine requirement was observed when these cultures were plated on the minimal medium solidified with Bacto-agar; many tiny

quots of the cultures into a series of tubes containing increasing amounts of L-phenylalanine. In all the tests, the growth responses observed were indistinguishable from those of inocula consisting of cells taken from the stock culture of strain 58-278 carried, with daily transfers, on yeast extract-peptone-agar slants. Thus, when growth studies were made with an inoculum consisting of cells taken from a bacterial culture at the end of the diphasic growth shown in Curve 2 or 3 of Fig. 1, the growth curves obtained were identical with those shown in Fig. 1. It appears, therefore, that bacteria present in cultures which give diphasic growth curves possess the phenylalanine requirement characteristic of strain 58-278.

Cell-free filtrates were then prepared from media in which the *phenylalanineless* mutant had been allowed to grow, and were tested for their ability to support the growth of a fresh inoculum taken from the stock culture of the mutant. The assay of filtrates for their ability to promote the growth of this strain could not be interpreted quantitatively. However, since the *phenylalanineless* mutant was never observed to grow during an incubation period of 90 to 100 hours, unless the medium contained either phenylalanine or bacterial filtrates, lack of growth in assay tubes was taken as an indication of the absence, from test filtrates, of the material which promoted growth. If detectable growth did not occur until late in the test period, *i.e.* after 50 to 60 hours, it was assumed that the filtrate under examination contained only small amounts of the active material, and early growth was thought to be indicative of large amounts of the active material. After all the assays, the bacteria which had grown up in the assay tubes were tested for their phenylalanine requirement.

All filtrates prepared from bacterial cultures which initially contained low concentrations of phenylalanine showed some activity, but high activity was found only in filtrates collected during the later stages of the secondary growth period. Filtrates of media which initially contained high concentrations of phenylalanine had only slight activity. Culture filtrates from strain 58 and from strain 58-6317, a mutant requiring biotin and proline, contained only traces of the active material. Thus, it would appear that during the growth of strain 58-278 some material is produced and accumulated in the medium and that this material can satisfy the requirement of strain 58-278 for phenylalanine.

As shown by the isotope experiment, the *phenylalanineless* strain has the

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colonies appeared on the plates within 24 hours. However, the same result was obtained when the organisms used for plating were taken from the stock slant of strain 58-278. Further tests of such colonies in liquid minimal medium and on minimal agar plates prepared with repurified agar showed that the colonies consisted of cells still requiring an exogenous source of phenylalanine for growth (*cf.* Ryan and Schneider (5)).

capacity for synthesizing tyrosine from the ammonia and glucose in the medium. In view of the close structural similarity between the two aromatic amino acids, the possibility must be considered that tyrosine, or some derivative of this amino acid, may be metabolized by the growing organism so as to provide a growth factor for the *phenylalanineless* mutant. The fact that tyrosine, when initially present in the medium, does not permit growth in the absence of phenylalanine and does not show any "sparing action" in the presence of suboptimal concentrations of phenylalanine (1) suggests that the active material is not a catabolic product of free tyrosine.

EXPERIMENTAL

Isolation of Isotopic Tyrosine—The composition of the minimal medium previously used (1, 2) was altered so that all the nitrogen would be supplied in the form of ammonia, and the nitrogen content of the medium was reduced to an extent still consistent with optimal growth. The medium used in this experiment had the following composition: per liter, 1.72 gm. of $(N^{15}H_4)_2SO_4$, 1.6 gm. of NaCl, 0.1 gm. of $MgCl_2 \cdot 7H_2O$, 1 mg. of $CaCl_2$, 3 gm. of K_2HPO_4, 1 gm. of KH_2PO_4, 1 ml. of trace elements solution (6); 5 gm. of glucose, 1 γ of biotin, and 2 mg. of L-phenylalanine. 250 ml. of medium were placed in each of four 2 liter Fernbach flasks and sterilized in the autoclave for 20 minutes at 15 pounds pressure. After inoculation with a heavy aqueous suspension of cells taken from a 24 hour slant of strain 58-278, the flasks were incubated at 30° for 24 hours, at which time the primary growth of the mutant had reached its maximum.

The bulk of the bacterial cells was removed by centrifugation and the resultant medium then was filtered through a bacterial filter. An aliquot of the cell-free filtrate was set aside for assay for tyrosine with strain 58-5030. 153 mg. of freshly recrystallized L-tyrosine were added to the filtrate, and another aliquot was removed for assay.

In the assays, samples of the cell-free filtrates (0.3 to 2 ml. of the original filtrate and 0.02 to 0.2 ml. of the "primed" filtrate) or of a standard solution of L-tyrosine were diluted to 5 ml. with water in Evelyn colorimeter tubes, and 5 ml. of double strength minimal medium (2) containing biotin (1 γ per liter) were added to each tube. Previous tests had shown that the stimulating action of the filtrates on the growth of the *tyrosineless* strain was masked by inhibitory material also present in the filtrate if more than 2 to 3 ml. of cell-free filtrate were used. However, with the smaller volumes, the activity observed was directly proportional to the volume of filtrate present in the assay tube.

After sterilization in the autoclave and inoculation with strain 58-5030, the tubes were incubated for 24 hours at 30°, and the extent of bacterial

growth was determined by measurement of the optical density of the cultures in an Evelyn colorimeter (filter No. 540). The results of the assay indicated the presence of 3.76 ± 0.8 mg. of tyrosine in the original filtrate and 150 ± 10.5 mg. of tyrosine in the primed filtrate.

For the isolation of tyrosine, the cell-free filtrate was concentrated *in vacuo* to approximately 200 ml., and phosphate was precipitated by the addition of barium hydroxide. The precipitate was filtered off and washed thoroughly with hot and cold water. The combined filtrate and washings were concentrated to about 25 ml. and adjusted to pH 5 with dilute HCl. The tyrosine which separated was collected and dissolved in a mixture of 10 ml. of water and 4 ml. of concentrated ammonia. This solution was filtered, and the tyrosine was reprecipitated by concentration of the ammoniacal solution *in vacuo*. After a second recrystallization from boiling water, a sample was set aside for N^{15} analysis, and the remainder of the tyrosine was crystallized again from boiling water.

The unwashed cells obtained on centrifugation of the culture were suspended in about 30 ml. of 6 N sulfuric acid. 149 mg. of L-tyrosine were added, and the mixture was refluxed for 20 hours. The sulfate in the hydrolysate was precipitated as $BaSO_4$, and the tyrosine was isolated and recrystallized in the manner described above. The results of the nitrogen and N^{15} analyses on the isolated samples are given in Table I.

Other Experiments with Phenylalanineless Strain—The details of a representative experiment showing the accumulation, in the medium, of material that stimulates the growth of the *phenylalanineless* strain are given below. 25 ml. of minimal medium (pH 7.0) containing biotin and 0.025 mg. of L-phenylalanine were dispensed into each of a series of 125 ml. Erlenmeyer flasks. After sterilization and inoculation, the flasks were incubated at 30° on a shaker. The extent of secondary growth under these conditions is much greater than that observed in tests employing Evelyn colorimeter tubes containing 10 ml. of medium; the final maximal density of cultures in flasks is about 0.70 rather than 0.25, the maximal density of cultures in tubes. When flasks are used, the extent of primary growth at suboptimal concentrations of phenylalanine is about the same as that in tubes. When the concentration of phenylalanine is above the optimum, primary growth is much greater in flasks than in tubes.

At intervals, flasks were removed from the shaker and the optimal density of the culture was measured. The bacteria were centrifuged and, if necessary, the pH of the cell-free solution was adjusted to 6.9 to 7.0 with dilute NaOH. Assays of the cell-free filtrates were carried out in Evelyn colorimeter tubes containing 0.1 to 2.5 ml. aliquots of filtrate diluted to 5 ml. with water and 5 ml. of double strength minimal medium (2) supplemented with biotin. Volumes of filtrate greater than 2.5 to 3.0 ml. could

not be used in the assays since, with those amounts, growth inhibition rather than growth stimulation was observed. The sparing action of filtrates on the phenylalanine requirement of strain 58-278 was tested by adding to the filtrate a small amount of L-phenylalanine. A filtrate that showed growth-promoting action always had the ability to increase the

TABLE II

Assay of Cell-Free Media from Phenylalanineless Strain

Filtrate No.	Incubation time before filtration	Optical density before filtration	Volume of filtrate used	Assay data		
				Optical density of assay tubes		
				24 hrs.	48 hrs.	96 hrs.
	hrs.		*ml.*			
I	24	0.042*	0.17	0	0	0
			0.50	0	0	0.071
			1.0	0	0.005	0.174
			1.7	0	0.005	0.154
			2.5	0	0.005	0.181
II	32	0.076	0.17	0	0	0.137
			0.50	0	0	0.143
			1.0	0 ·	0.004	0.161
			1.7	0.011	0.049	0.179
			2.5	0.025	0.114	
III	39	0.155	0.17	0	0	0
			0.50	0	0	0.092
			1.0	0.009	0.015	0.155
			1.7	0.019	0.154	
			2.5	0.037	0.172	
IV	48	0.690†	0.17	0.037	0.077	0.155
			0.50	0.154	0.179	
			1.0	0.209		
			1.7	0.222		
			2.5	0.212		
			Control, minimal medium	0	0	0

* Approximate maximum of primary growth phase.
† End of secondary growth phase.

amount of primary growth obtained in the presence of a suboptimal amount of phenylalanine.

Some of the data obtained in this experiment are given in Table II. It will be seen that the active material appears to be present in largest amounts in filtrates collected at the end of the secondary growth phase. A filtrate prepared from a culture of strain 58-278 at the end of primary growth in the presence of an optimal concentration of L-phenylalanine and a filtrate

from a culture of strain 58 grown in the absence of L-phenylalanine had about the same activity as Filtrate I in Table II. When such cultures of strains 58-278 and 58 were incubated for 24 hours after maximal growth had been attained, there was only a very slight increase in the amount of active material in the culture filtrates.

SUMMARY

A phenylalanine-requiring mutant of *Escherichia coli* strain K-12 has been found to produce a growth factor for a tyrosine-requiring mutant of the same parent strain and for *Lactobacillus arabinosus*. When the *phenylalanineless* strain is cultured in minimal medium containing L-phenylalanine as the only amino acid and $N^{15}H_4^+$, it synthesizes and elaborates tyrosine containing N^{15} into the medium. Since the total quantity of tyrosine in the bacterial cells and in the culture filtrate is much greater than the quantity of L-phenylalanine supplied in the medium, the carbon skeleton of tyrosine cannot be entirely derived from the exogenous phenylalanine.

The *phenylalanineless* strain exhibits diphasic growth curves when it is cultured for long periods in minimal medium containing suboptimal concentrations of L-phenylalanine. The first phase of such curves represents growth due to the utilization of phenylalanine supplied in the medium, and the second phase growth in the absence of an exogenous source of phenylalanine. Evidence is presented to demonstrate that the secondary growth is not the result of a spontaneous loss, by the bacteria, of their phenylalanine requirement, but that the mutant elaborates into the medium, a substance which can serve as a growth factor in place of phenylalanine.

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A SYNTHESIS OF 2,3-DIPHOSPHO-D-GLYCERIC ACID

By ERICH BAER

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In 1925, Greenwald (1) isolated from the erythrocytes of dog and pig blood a previously unknown organic phosphate and established its constitution as that of a 2,3-diphosphoric acid ester of *l*-glyceric acid (DPGA). This compound has ever since caused speculation as to its biological significance. Recently, Sutherland, Posternak, and Cori (2) have shown that phosphoglyceric acid mutase, the enzyme which catalyzes the reaction, 3-phosphoglyceric acid $\rightleftharpoons$ 2-phosphoglyceric acid (3), requires the presence of catalytic amounts of 2,3-diphosphoglyceric acid for its activity.

$$
\begin{array}{ccc}
\text{COOCH}_3 & & \text{COOCH}_3 \\
| & & | \\
\text{H}-\text{C}-\text{OH} & \xrightarrow[\text{Pyridine}]{(\text{C}_6\text{H}_5\text{O})_2\text{POCl}} & \text{H}-\text{C}-\text{O}-\text{PO}(\text{OC}_6\text{H}_5)_2 \xrightarrow[\text{Acetic acid}]{\text{Pt, H}_2} \\
| & & | \\
\text{H}_2\text{C}-\text{OH} & & \text{H}_2\text{C}-\text{O}-\text{PO}(\text{OC}_6\text{H}_5)_2 \\
\text{(I)} & & \text{(II)}
\end{array}
$$

D-Glyceric acid
methyl ester

$$
\begin{array}{ccc}
\text{COOCH}_3 & & \text{COONa} \\
| & & | \\
\text{H}-\text{C}-\text{O}-\text{PO}_3\text{H}_2 & \xrightarrow[\text{hydroxide}]{\text{Sodium}} & \text{H}-\text{C}-\text{O}-\text{PO}_3\text{Na}_2 \xrightarrow{\text{Barium hydroxide}} \\
| & & | \\
\text{H}_2\text{C}-\text{O}-\text{PO}_3\text{H}_2 & & \text{H}_2\text{C}-\text{O}-\text{PO}_3\text{Na}_2 \\
\text{(III)} & & \text{(IV)}
\end{array}
$$

2,3-Diphospho-D-glyceric acid (pentabarium salt)*

* 2,3-Diphospho-L-glyceric acid and 2,3-diphospho-DL-glyceric acid can be obtained by the same sequence of reactions with either L- or DL-glyceric acid methyl ester, respectively, as starting materials.

A procedure has now been developed which not only permits the synthesis of 2,3-diphospho-D-glyceric acid in a configurationally pure state but also serves to establish unambiguously the steric relationship of the synthetic and of the natural (Greenwald's) diphosphoglyceric acid to the glyceraldehydes and thus to the various intermediary products of fermentation and glycolysis.

The synthesis of 2,3-diphospho-D-glyceric acid was carried out as follows (see the reaction scheme): D-glyceric acid methyl ester (I), obtained

by oxidation of D-glyceraldehyde and esterification of the D-glyceric acid, was phosphorylated by means of diphenylphosphoryl chloride in the presence of pyridine. The phosphorylation product, 2,3-bis(diphenylphosphoryl)-D-glyceric acid methyl ester (II), was isolated and, without further purification, freed from its phenyl groups by catalytic hydrogenolysis. The resulting 2,3-diphospho-D-glyceric acid methyl ester (III) was saponified with dilute alkali and the 2,3-diphospho-D-glyceric acid (IV) was isolated in the form of its water-insoluble pentabarium salt. After purification by precipitation the analytical figures for the barium salt agreed well with those calculated for the diphosphoglyceric acid pentabarium salt (trihydrate).

The specific rotations of the free acid and of its pentasodium salt were found to be similar to those reported by Greenwald (1) and Jost (4) for the natural DPGA. In order to ascertain whether the levorotation of the synthetic DPGA was authentic or was caused by the presence of the levorotatory D-3-phosphoglyceric acid, the DPGA of several independently prepared barium salts was liberated by the addition of sulfuric acid and was converted successively into the lead, brucine, and barium salts, as described by Jost (4). Both the brucine and the barium salts were obtained in an analytically pure state.

Brucine salt.   Calculated, P 2.77; found 2.76
Barium salt trihydrate.   Calculated. C 5.70, H 0.95, P 9.83, Ba 54.37
Found.   " 5.78, " 1.34, " 10.0,   " 54.21

The regenerated DPGA, as well as its sodium salt, exhibited the same optical activity as before. The absence of D-3-phosphoglyceric acid was further confirmed by the non-appearance of an enhanced negative rotation on the addition of ammonium molybdate.

The synthetic levorotatory DPGA, as a derivative of D-glyceric acid, is related to D-glyceraldehyde and therefore belongs stereochemically to the D series. The same steric classification must be assigned to the natural levorotatory DPGA of Greenwald. The isolation of D-glyceric acid from the acid hydrolysates of the natural DPGA (1) is in agreement with this designation.

EXPERIMENTAL

D-*Glyceric Acid Methyl Ester*—1,2,5,6-Diacetone D-mannitol (5) was oxidized with lead tetraacetate and the cleavage product, acetone D-glyceraldehyde, was isolated by fractional distillation *in vacuo* (6). The acetone D-glyceraldehyde was hydrolyzed with dilute acetic acid, the glyceraldehyde isolated (7) as a viscous oil, oxidized by means of bromine (8), and the resultant D-glyceric acid esterified with methanol (8). The D-glyceric acid methyl ester was obtained in an over-all yield of 54 per cent

based on diacetone D-mannitol. $[\alpha]_D^{26} = +4.7°$ (in substance); b.p. (9 mm.) 114–116°.

2,3-Bis(diphenylphosphoryl)-D-glyceric Acid Methyl Ester (II)—To a solution of 12.0 gm. of D-glyceric acid methyl ester (I) in 210 ml. of dry pyridine,[1] 60.0 gm. of pure diphenylphosphoryl chloride (9) were added under anhydrous conditions and with cooling in ice. Pyridine hydrochloride separated almost immediately. The mixture was kept for 1 hour in ice, 24 hours at +8°, and a further 24 hours at room temperature (23–25°). 1 hour before the end of this period, 2.4 ml. of water were added to destroy the excess of diphenylphosphoryl chloride. The solution was concentrated *in vacuo* (8 to 10 mm.) at a bath temperature of 25–30° as far as possible to remove the major part of the pyridine. The viscous residue was then shaken with 150 ml. of water and 900 ml. of ether. The layers were separated and the water layer extracted with 100 ml. of ether. The combined ether layers were washed in quick succession with two 300 ml. portions of 2.5 N sulfuric acid, two 90 ml. portions of a half-saturated solution of sodium bicarbonate, and two 60 ml. portions of water. The ether solution was then dried over anhydrous sodium sulfate, filtered, and the sulfate washed with anhydrous ether. The combined filtrates were first concentrated under normal pressure and then kept for several hours *in vacuo* (0.05 mm.) at a bath temperature of 30–32° to remove all organic solvent. The 2,3-bis(diphenylphosphoryl)-D-glyceric acid methyl ester (II), a water-clear, viscous liquid, weighed 45.0 gm. (75 per cent yield) and was sufficiently pure for further treatment. $n_D^{26} = 1.5540$, $n_D^{23} = 1.5559$, $[\alpha] = +0.18° \pm 0.03°$ (1 dm. tube, in substance).

Analysis—$C_{23}H_{26}O_{10}P_2$ (584).   Calculated P 10.61; found, 10.50

2,3-Diphospho-D-glyceric Acid Pentabarium Salt. Catalytic Hydrogenolysis of Phenyl Ester—A solution of 11.68 gm. of 2,3-bis(diphenylphosphoryl)-D-glyceric acid methyl ester (II) in 140 ml. of glacial acetic acid was shaken with 1.4 gm. of platinum oxide (Adams' catalyst) in an atmosphere of hydrogen under slightly positive pressure until the absorption of hydrogen practically ceased (approximately 2 hours). The hydrogen was replaced by nitrogen, and the solution filtered and concentrated *in vacuo* as far as possible at a bath temperature of 30–35°. The residue was taken up in 30 ml. of water and the solution was concentrated again *in vacuo*. This procedure was repeated once more.

Saponification of Methyl Ester—To the above residue, consisting mainly of 2,3-diphospho-D-glyceric acid methyl ester (III), 1 N sodium hydroxide was added until the solution was just alkaline to phenolphthalein. An additional one-third of the amount of 1 N sodium hydroxide which was

[1] The pyridine was dried by refluxing over barium oxide.

required for the alkalization was then added, and the solution was kept at 35° for a period of 20 hours.

Removal of Inorganic Phosphoric Acid—The hydrolysate was filtered, made neutral to litmus with concentrated acetic acid, and finally was made faintly alkaline to phenolphthalein with concentrated ammonia. Then ammoniacal magnesia mixture[2] was added cautiously until in a centrifuged sample no further precipitation was elicited. The precipitate was removed and 10 N sodium hydroxide was added until a centrifuged sample failed to give a precipitation on further addition of alkali. The magnesium hydroxide was centrifuged,[3] washed in the centrifuge with a small amount of water, and the combined supernatant solutions were made neutral to litmus with concentrated acetic acid.

Preparation of Barium Salt—To the solution of the sodium salt of the diphosphoglyceric acid was added a filtered,[4] lukewarm aqueous solution of baryta (15 gm. of $Ba(OH)_2 \cdot 8H_2O$ per 75 ml. of water) until in a centrifuged sample the supernatant liquid was distinctly alkaline to phenolphthalein and gave no precipitate on further addition of baryta. The barium salt was filtered with suction, well washed with water, resuspended in 150 ml. of water, brought into solution by adding cautiously 12 N hydrochloric acid, and reprecipitated with a lukewarm solution of baryta as described above. The barium salt was filtered, thoroughly washed with water, and dried *in vacuo* (0.005 mm.) over phosphorus pentoxide, first at room temperature and then at 100° (1 hour); it weighed 7.05 gm. (55.6 per cent of the theoretical yield).

Analysis—$(C_3H_3O_{10}P_2)_2Ba_5 + 3H_2O$ (1263)

           Calculated.   C 5.70, H 0.95, P 9.83, Ba 54.37
           Found.   " 5.90, " 1.11, " 10.06, " 54.70

Optical Activity of 2,3-Diphospho-D-glyceric Acid—The pentabarium salt, with a known content of barium, was suspended in 10 times its weight of water, and was triturated in a mortar with the theoretical amount of 1 N sulfuric acid. The barium sulfate was removed by centrifugation, the supernatant liquid concentrated *in vacuo* (bath 30–35°), and the residue made up to a known volume. The content of DPGA was ascertained by phosphorus determination. In six independent preparations the specific rotation, $[\alpha]_D$, for the free acid ranged from $-1.7°$ to $-2.3°$ (c, 6.4 to 17 in water or 1 N HNO_3).[5] Previously reported values are as follows: (a) by

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[2] Prepared as previously described (10), except that only 10 ml. of concentrated ammonia per 100 ml. of magnesia mixture were added.

[3] If the magnesium is not removed at this stage it will contaminate the barium salt.

[4] The filtration of the baryta solution and the neutralization of the DPGA were carried out under a protective layer of toluene.

[5] One sample of DPGA was obtained from a barium salt which had gone through the procedure reported by Jost (4) for the isolation of DPGA from natural sources.

Greenwald (1) $[\alpha]_D = -3.36°$ (c, 27.7), and (b) by Jost (4) $[\alpha]_D = -2.3°$ (c, 8.3).

Optical Activity of Pentasodium Salt of 2,3-Diphospho-D-glyceric Acid—Four of the aqueous solutions of DPGA which were obtained as described above from four independently prepared barium salts of synthetic DPGA were made distinctly alkaline to phenolphthalein by the addition of 50 per cent (by weight) sodium hydroxide solution and their contents of DPGA were ascertained by phosphorus determination. The specific rotation, $[\alpha]_D$, calculated on the basis of the content of pentasodium salt, was invariably $-3.5°$ (c, 8.3 to 20);[5] $[\alpha]_D$, calculated on the basis of the content of free acid, was $-4.9°$ to $-5.0°$ (c, 5.9 to 14).[5] The value reported by Greenwald (1) is $[\alpha]_D^{17} = -4.01°$ (c, 27.7).

SUMMARY

1. A synthesis of 2,3-diphospho-D-glyceric acid[6] (pentabarium salt) is described.

2. Both the synthetic acid and its sodium salt are levorotatory. Their optical rotations agree fairly well with those reported by Greenwald and by Jost for the naturally occurring 2,3-diphosphoglyceric acid.

3. The D configuration of the natural 2,3-diphosphoglyceric acid was confirmed.

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[6] Dr. Carl F. Cori has informed the author that Dr. E. Sutherland in his laboratory has compared the natural and synthetic 2,3-diphospho-D-glyceric acid and has found in preliminary tests that both compounds behave identically with phosphoglyceric mutase. The author wishes to express his thanks to Dr. Sutherland.

FURTHER STUDIES ON THE KINETICS AND DETERMINATION OF ALDOLASE

BY ALEXANDER L. DOUNCE, SHIRLEY R. BARNETT, AND G. THANNHAUSER BEYER

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A new method for the determination of aldolase was published from this laboratory in 1948 (1). Subsequently Sibley and Lehninger (2) published a different method for determining the same enzyme. The kinetics described by the latter authors differed in most respects from those published by us, as did some of the analytical results obtained by determining the enzyme in tissues. One of the most glaring discrepancies in the latter category was the difference in the ratio of the activity of aldolase in liver to that of aldolase activity in muscle. The present paper is the outcome of many experiments designed to show the cause of the differences referred to above and to decide what method may be the most suitable for aldolase determination in tissues. Muscle and liver have been studied thoroughly, but a few analyses of some other tissues have also been included to show the order of magnitude of results to be expected.

EXPERIMENTAL

Preparation of Substrate—A preparation of crude barium fructose diphosphate, kindly furnished by Dr. Henry Scherp, and preparations purchased from the Schwarz Laboratories, Inc., were purified according to the following procedure. 1 part by weight of the crude dibarium salt was extracted at 0° with 40 parts by volume of distilled water, with stirring, for 1 hour at pH 6.5. The supernatant (containing barium hexose diphosphate) was recovered by centrifugation in the cold and the residue was reextracted eight times at 0° by use, for each extraction, of approximately two-thirds of the volume used in the preceding extraction. All extracts were then combined and decolorized with acid-washed norit. The dibarium fructose diphosphate was precipitated from the combined decolorized extract at room temperature and about pH 7.2 by the addition of 1 volume of ethyl alcohol. After centrifugation at room temperature, the precipitated material was washed with alcohol and ether, dried, and weighed.

Next, the partially purified dibarium fructose diphosphate was converted to the acid barium salt according to Neuberg *et al.* (3). 1 part

by weight of the dibarium salt was dissolved in 10 parts by volume of distilled water by adding dropwise the minimal amount of glacial acetic acid needed to cause solution of the material. Then sufficient 5 N sulfuric acid was added in the cold to remove one-half the calculated amount of barium. (This step was found to be necessary to avoid subsequently precipitating a mixture of mono- and dibarium salts.) The barium sulfate was removed by centrifugation in the cold and the supernatant was immediately poured into 6 volumes of alcohol to precipitate the acid barium salt. The latter was washed with alcohol and ether, dried, and weighed. The acid barium salt was then dissolved in ice water and sufficient barium acetate was added to regenerate the dibarium salt. The pH was adjusted to 7.2 and the precipitated dibarium salt was recovered by centrifugation at room temperature. This purified dibarium salt was washed with 50 per cent alcohol, 95 per cent alcohol, and ether, and dried. Substrate solutions were subsequently prepared by conversion of the dibarium to the disodium salt.

By the method of purification described above, about 20 per cent of purified dibarium salt was recovered from one batch of Schwartz dibarium fructose diphosphate.

pH Optimum of Enzyme—Sibley and Lehninger (2) claim, in agreement with Herbert *et al.* (4), that the optimal pH for aldolase is in the alkaline pH range, in the neighborhood of pH 8.5 to 9.0. However, the former authors do not state in their article whether purified aldolase or a tissue homogenate such as liver was used in determining this pH optimum. Moreover, different buffers are employed in plotting the same pH curve, a practice which is not to be recommended unless it is proved that all of the buffers affect the activity of the enzyme to the same degree. We have reinvestigated the pH optimum shown by crystalline muscle aldolase, using the method as originally described by Sibley and Lehninger (2), and also using modifications of this method in which different buffers or no buffer at all was employed. The results of some of these experiments are shown in Fig. 1. We conclude that in all cases the pH optimum is in the neighborhood of 7.2 except when the trishydroxymethylaminomethane recommended by Sibley and Lehninger is used as buffer. In the latter case, a curve with two peaks is obtained with the usual optimum at pH 7.2 and a second optimum at approximately pH 8.3. This type of curve is not an artifact since it has been observed repeatedly.

As can be seen from Fig. 1, in which the same amount of enzyme was used in all cases, the apparent activity of the enzyme is greatly enhanced by using trishydroxymethylaminomethane as buffer.

In attempting to account for the second pH optimum which occurs with crystallized aldolase at about pH 8.5 in the presence of trishydroxymethyl-

aminomethane buffer, attention has been given to the possibility that the buffer might act as a catalyst in splitting substrate to trioses in alkaline solution. Catalysis of a reaction of a similar type is reported to be characteristic of primary or secondary amines (5). We have found that, if the substrate (fructose diphosphate) is incubated with the trishydroxymethylaminomethane buffer, chromogenic material which gives color with
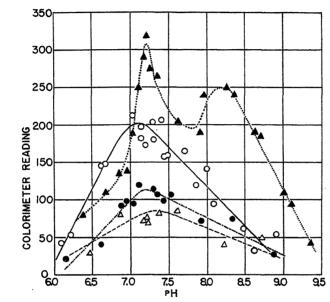
FIG. 1. pH optimum of crystallized aldolase. ▲, trishydroxymethylaminomethane buffer; ○, collidine buffer; ●, no buffer; △, phosphate buffer. In order to obtain many points on each curve, analyses were carried out over a period of several days, usually with several new dilutions of the stock enzyme solution. For this reason there is more scatter than will occur when points are obtained on 1 day with the same dilution of the stock solution and the same buffer substrate solution. The same degree of dilution of the stock solution was used in all cases. The Sibley and Lehninger method of analysis (2) was used in all cases.

the dinitrophenylhydrazine of Sibley and Lehninger is produced in increasing amounts as the pH is increased from 7.5 to 9.0, provided that controls are employed in which the buffer is added to the incubated substrate immediately before color development. However, the rate of production of chromogenic material, judging from the limited data in Fig. 2, seems to be linear, and the amount of chromogen produced at pH 8.6 is insufficient in amount to account for the height of the second pH optimum referred to above (see Fig. 1). Hence the buffer may, in addition, have an activating effect on the enzyme in the neighborhood of this pH.

The abnormal pH-activity curve which is obtained with liver homogenate as the enzyme source in the presence of buffers other than trishydroxymethylaminomethane cannot be explained at the present time except as being probably due to the presence of interfering substances in the homogenate which are not sufficiently diluted to be negligible in effect.

We have also estimated the pH optimum of aldolase, using the Barker-Summerson color test as modified by us (1), and have obtained results that are in substantial agreement with those just reported. In fact, it can be stated as the result of a number of experiments with the aldolase reaction carried out under different conditions in which color was produced both by the method of Sibley and Lehninger (2) and by the modified Barker-Summerson procedure (1) that color development by one method is closely proportional to that by the other.
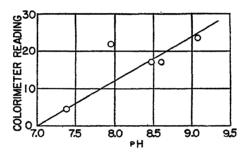
FIG. 2. Effect of pH on chromogen production by the action of trishydroxymethylaminomethane buffer on substrate without enzyme. The procedure was that of Sibley and Lehninger, except that 0.5 ml. of buffer was employed. Blanks with buffer added after the incubation have been subtracted.

We have also determined the pH optimum for aldolase, using suitably diluted muscle or liver homogenate and buffers other than the trishydroxymethylaminomethane buffer, and have found that muscle homogenate gives curves similar to those obtained with solutions of crystallized aldolase. Fig. 3 shows the results with collidine buffer. On the other hand, liver homogenate always gave curves with a broad optimal range extending from pH 7.0 to 8.6, as found by Sibley and Lehninger (2), or in some cases curves with a second peak at approximately pH 8.6.

We thus conclude that our original curve for the pH optimum of crystallized aldolase was essentially correct, and that assay of the enzyme at pH 8.6 is not to be recommended. That the pH optimum originally published by us is slightly lower than 7.2 might be accounted for by the facts that in our original method a higher substrate concentration was used without hydrazine as a fixative and that the substrate preparations originally used were not extensively purified.

Temperature Optimum—Sibley and Lehninger (2) stated that the temperature optimum for aldolase is above 50°, apparently relying on the work of Herbert *et al.* (4) to substantiate their statement. Their own reaction was carried out at 38°, which they state is considerably below the temperature optimum for aldolase.
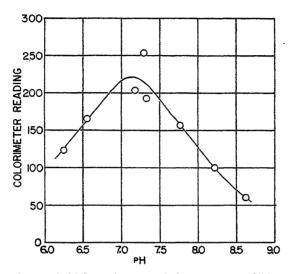
Fig. 3. pH optimum of aldolase of rat muscle homogenate. Sibley and Lehninger method of analysis (collidine buffer).

In checking this statement we have used the method of Sibley and Lehninger, modified by using collidine buffer at pH 7.2, which we take to represent optimal pH in the presence of a suitable buffer. Fig. 4 shows the curve for the temperature optimum obtained in this manner, and also the results of a few determinations carried out at pH 8.6 by the unmodified method of Sibley and Lehninger (2). It is clear that in both cases aldolase activity decreases rapidly at temperatures above 30°. The descending portion of the graph showing the temperature optimum at pH 8.6 has also been confirmed, as can be seen from a third curve in Fig. 4 in which higher ordinates are recorded. In addition, a fourth curve is included to illustrate the result of heating the enzyme at various temperatures at pH 7.1 to 7.2 in the presence of collidine buffer and hydrazine without substrate for 30 minutes and subsequently carrying out determinations of activity at 25° by our modification of the procedure of Sibley and Lehninger.[1] In

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[1] Our modification of the method of Sibley and Lehninger (2) is as follows: To a 15 ml. centrifuge tube, add 0.25 ml. of 0.05 M disodium fructose diphosphate adjusted approximately to pH 7.2. Then add 1.0 ml. of 0.1 M collidine buffer of pH 7.2, 0.25 ml. of 0.56 M hydrazine sulfate adjusted to pH 7.2, and 0.25 ml. of 0.002 M iodoace-

this type of experiment the enzyme is inactivated by heating before being determined at a temperature at which it is completely stable. When this technique was employed, the decrease in enzymatic activity also occurred at approximately 30°.

It is not necessarily true, however, that the temperature optimum for a crystallized enzyme should be the same as that of the enzyme in a crude
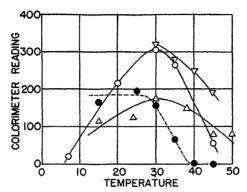
FIG. 4. Temperature optimum of crystallized aldolase. ○, pH 7.2, modified Sibley and Lehninger method (with collidine buffer at pH 7.2); △, ▽, pH 8.6, Sibley and Lehninger method (with trishydroxymethylaminomethane buffer at pH 8.6); ●, effect of heating aldolase alone for 30 minutes at various temperatures and then determining at 25° by the modified Sibley and Lehninger method (with collidine buffer at pH 7.2).

homogenate. Fig. 5 shows the effect of temperature on the aldolase reaction as measured by the modified reaction of Sibley and Lehninger for muscle and liver homogenate. It will be noted that muscle homogenate gives an activity-temperature curve similar to that of the pure enzyme, with an optimum at about 35°, while the activity of liver homogenate increases steadily throughout the temperature range studied. This is probably due to the necessarily lower dilution of liver homogenate which

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tate. Next add 0.55 ml. of distilled water. Finally, bring the mixture to 25°, add 0.2 ml. of properly diluted enzyme solution or homogenate, and incubate for 30 minutes. Stop the reaction by adding 2.0 ml. of 10 per cent trichloroacetic acid, centrifuge if any precipitate is visible, and use a 1 ml. aliquot for analysis according to the directions of Sibley and Lehninger (2). The blank determination is made by mixing all materials except substrate, incubating, and then adding the trichloro-acetic acid followed by the substrate. Rat muscle homogenate should ordinarily be made up to a final dilution of about 1 gm. of muscle (wet weight) in 1000 ml. of homogenate, and for rat liver the final dilution is usually about 1 gm. of liver (wet weight) to 50 ml. of homogenate. Required dilutions for other tissues can be calculated from the data in Table I.

permits protective action by proteins and other materials in the homogenate against denaturation. Muscle homogenate, on the other hand, is diluted so much that protective action against denaturation of the aldolase presumably is minimal.

It is now apparent that if the aldolase reaction is carried out at 38° the ratio of the activity of aldolase in muscle to the activity of the enzyme in liver (on a dry weight basis) will be about 3-fold too high, as judged from
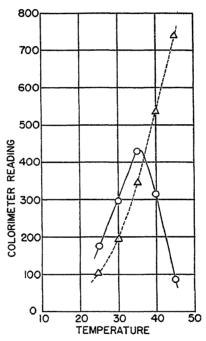
FIG. 5. Temperature optimum for muscle and liver homogenate. $\bigcirc$, muscle homogenate; $\triangle$, liver homogenate. Modified Sibley and Lehninger analysis used (with collidine buffer at pH 7.2).

the curves in Fig. 5. In determining the aldolase content of tissues, a temperature no higher than 30° should be used; we have, however, adhered to the temperature of 25° to allow a reasonable margin of safety.

Effect of Substrate Concentration—Sibley and Lehninger, in agreement with the work of Herbert *et al.* (4), state that the dissociation constant (K_m) for aldolase is in the neighborhood of 0.001, which is contrary to our value of 0.009. We have repeated the work of Sibley and Lehninger (with crystallized aldolase and collidine buffer at pH 7.1 to 7.2), using their otherwise unmodified procedure, and have obtained a K_m value lying be-

tween 0.001 and 0.002, which thus agrees approximately with their value. However, when hydrazine was omitted from the reaction mixture, the enzyme was not nearly saturated with substrate at 0.056 M substrate concentration, a result which is in qualitative agreement with our original data. (It was not considered worth while to carry out sufficient experiments without hydrazine to check our original K_m value quantitatively.) These experiments would appear to indicate either that the reaction products inhibit the enzyme to a high degree or that hydrazine enters into the enzyme-substrate complex, causing a decreased dissociation of the enzyme. In the absence of known cases illustrating the latter possibility, it would seem safer to assume that the former is the correct one, although this means that the affinity of enzyme for products must be quite high in comparison to its affinity for the substrate.

We therefore conclude that the K_m value reported by Herbert *et al.* (4) and by Sibley and Lehninger (2) is more nearly correct than that previously reported by us (1).

Choice of Method for Determining Aldolase—We have made a number of determinations of aldolase with crystalline enzyme, muscle homogenate, and liver homogenate, in which parallel determinations of color were made with the Sibley and Lehninger procedure and with our modification of the Barker-Summerson procedure (1). These determinations have demonstrated that equally reliable color determinations can be made with either method. Our original method (1), in the absence of fixative, made use of high substrate concentrations in order to attain an approximately linear relationship between amount of enzyme added and amount of color observed. We have found that this original method can be modified by using a 0.01 M substrate concentration in the presence of hydrazine and 0.05 M collidine buffer so as to yield a considerably improved method with greatly lowered blank values and with a linear relationship between amount of enzyme used and amount of color produced, even in the case of liver homogenate. The pH employed is 7.2 and the temperature is 25°.[2]

[2] Our modified method is carried out as follows: Add 0.5 ml. of 0.01 M disodium fructose diphosphate, adjusted approximately to pH 7.2, to a 15 ml. centrifuge tube. Then add 0.1 ml. of 0.4 M collidine buffer (of pH 7.2) and 0.1 ml. of 0.056 M hydrazine sulfate adjusted to pH 7.2. Following this, 0.1 ml. of 0.002 M iodoacetate is added. This mixture is brought to 25° and 0.2 ml. of enzyme or properly diluted tissue homogenate is finally added. After 15 minutes the reaction is stopped by adding 4.0 ml. of 8 per cent trichloroacetic acid, and if any precipitate is visible it is removed by centrifugation. A 1 ml. aliquot of the supernatant is used for analysis by the modified Barker-Summerson method as previously described by us (1). (Note, foot-note 3 of Dounce *et al.* (1) should be changed to read, "1.5 gm. of *p*-hydroxydiphenyl (Eastman grade) are dissolved in 100 ml. of 0.5 per cent NaOH. . .".) The blank determination is made by mixing all materials except enzyme, incubating, and then

Our modification of the method of Sibley and Lehninger[1] also seems to be reliable. It is slightly less sensitive than our modified method, but is free from interference by traces of lactic acid from the fingers.

Ratio of Aldolase Concentration in Liver to Aldolase Concentration in Muscle—We reported that the ratio of the aldolase concentration in muscle to that in liver was approximately 39:1, while Sibley and Lehninger stated that this ratio should be in the neighborhood of 6:1. The preceding experiments in this paper appear to demonstrate that the determination used by Sibley and Lehninger yielded unduly high values from liver homogenate compared with muscle homogenate or purified enzyme, owing chiefly to their choice of temperature. On the other hand our original results gave values for liver which were somewhat too low, presumably because of nonlinear curves caused by lack of a fixative for the triose phosphates. Using hydrazine as a fixative, we are now able to obtain linear curves at a much lower substrate concentration than was previously employed by us, and we have concluded that the correct ratio of the concentration of aldolase in muscle to its concentration in liver is about 18:1 instead of 39:1, as previously reported by us, or 6:1 as reported by Sibley and Lehninger.

Using the modified method of Sibley and Lehninger,[1] we now obtain for the ratio of the concentration of aldolase in muscle to concentration of aldolase in liver a value of approximately 16:1. Thus there is fairly good agreement between the two modified methods, although this agreement may not yet be exact.

The results of determining aldolase in various tissues of the normal rat by the two modified methods are given in Table I, together with results calculated from our original paper (1) and the paper of Sibley and Lehninger (2).

Substrate Specificity—If the experiments of Meyerhof and collaborators (6) on the formation of fructose-1-phosphate by aldolase action are correct, it seems obvious that aldolase would also catalyze the reverse reaction by acting on fructose-1-phosphate itself. We have been unable to obtain fructose-1-phosphate, but we have observed a catalytic splitting with fructose-6-phosphate[3] as measured by the modified Sibley and Lehninger method.[1] The pH-activity curve for this substrate is shown in Fig. 6. This result is contrary to that of Herbert *et al.* (4) who may have used

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adding the trichloroacetic acid, followed by the enzyme. At least in the case of liver and muscle, iodoacetate was found to be unnecessary in the presence of hydrazine, since it makes little or no difference in the results when it is omitted either in this method or in the method described in foot-note 1. Homogenates are diluted as described in foot-note 1.

[3] We owe thanks to Dr. Gerhardt Schmidt for furnishing us with fructose-6-phosphate.

TABLE I

Aldolase Activities of Various Rat Tissues

Organ	Relative activity, dry weight basis (muscle taken as 100)			
	Dounce-Beyer original (1)	Dounce-Beyer modified	Sibley-Lehninger modified	Sibley-Lehninger original (2)
Muscle	(6)	55		(12)
		78		
		105	85*	
		106*	98	
		117	99†	
		119‡	118‡	
		120		
	100	**100**	**100**	**100**
Liver	(4)	3.8		(11)
		3.5		
		5.4	5.2*	
		5.6	5.4	
		6.1	6.6†	
		6.3	7.7‡	
		6.4		
	2.6	**5.5**	**6.4**	**16.3**
Kidney	(4)	4.5	(1)	(12)
		5.3		
	5.5	**4.9**	**6.5**	**10.0**
Spleen	(1)	3.3	(1)	(11)
		3.9		
	1.6	**3.6**	**3.7**	**6.4**
Pancreas		0.36		(8)
		1.04		
		0.70		**0.8**
Walker carcinoma 256	(2)	12.1		
		15.2		
	19.4	**13.7**		
Nuclei of normal rat liver	(6)	2.4		
		2.6		
	1.6	**2.5**		

The above determinations by the two modified methods represent averages of duplicate determinations on the same homogenate. The figures in parentheses represent the number of determinations; the bold-faced figures represent the averages of the determinations listed.

* From the same animal. † From the same animal. ‡ From the same animal.

too high a pH to obtain any splitting. However, we have been unable to obtain a measurable action of aldolase on fructose, glucose, or glucose-6-phosphate over a wide range of pH values.

Relationship between Color Production in Our Original Method and in Our Modified Method—Crystalline aldolase was determined by the original method (1) and by our modified method.[2] It was found that the new method is more sensitive by a factor of approximately 2.5. This gain in
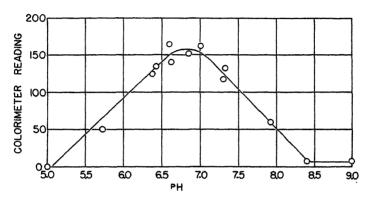
Fɪɢ. 6. pH optimum for crystallized aldolase with fructose-6-phosphate as substrate. Sibley and Lehninger type of analysis, with collidine buffer.

sensitivity is accompanied by a considerable lowering of the blank reading, which removes a disadvantage present in our original method (1).

DISCUSSION

The experiments outlined in this paper essentially confirm most of our previously published conclusions about the kinetics of aldolase action.[4] However, the K_m value previously reported by us appears to have been erroneously high, owing to inhibition of the enzyme by the reaction products.

Sibley and Lehninger state that their method for determining aldolase was standardized against a method of known reliability, which in essence is that used by Herbert *et al.* (4), possibly with more control values. The correlation obtained by these investigators between color production in their method and the formation of alkali-labile phosphate as the result of aldolase action was offered in support of this statement. However, it is obvious that the production of alkali-labile phosphate must be affected by temperature and type of buffer in the manner illustrated in this paper by means of the colorimetric determinations. Moreover, the formation of

[4] Our work throws doubt upon the pH optimum of pea aldolase (7), which has been determined by the method of Herbert *et al.* (4).

alkali-labile phosphate may be subject to the possible additional errors of non-enzymatic hydrolysis of the substrate as the pH increases, especially at high temperature, and hydrolysis of the substrate by alkaline phosphatase. Thus in the case of enzyme assays it is difficult to claim a "standard method," and the empirical method adopted must be based on the most thorough study of the kinetics of the enzyme concerned. This we have attempted to provide in the case of aldolase.

The data in Table I, which show good agreement between our own modified method[2] and the modified method of Sibley and Lehninger,[1] nevertheless may indicate that the agreement is not quite perfect, since, for example, the ratio of muscle to liver aldolase was worked out by both methods on identical homogenates, and the resulting slight difference in ratio occurred on repetition of the experiment with tissues from different animals.

One possible cause for the remaining small discrepancy might be a slight conversion of glyceraldehyde-3-phosphate into dihydroxyacetone phosphate, in spite of the presence of hydrazine. The method of Sibley and Lehninger gives higher color values for dihydroxyacetone than for glyceraldehyde, whereas our color reaction seems to be about the same for both of the triose phosphates (1).

SUMMARY

1. The kinetics of aldolase as originally determined by Dounce and Beyer (1) have been confirmed except for the K_m value, which was probably too high.

2. Two improved simple methods for determining aldolase are reported, which are based upon the methods of Dounce and Beyer (1) and of Sibley and Lehninger (2). These modified methods now give ratios of aldolase concentrations among various animal tissues which are in good agreement.

3. The ratios of aldolase concentrations among various animal tissues originally reported by Dounce and Beyer (1) appear for the most part to be correct. However, the ratio of aldolase concentration in muscle to aldolase concentration in liver now is found to be about 18:1 instead of 38:1, as originally reported (1), or 6:1, as given by Sibley and Lehninger (2).

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THE PATH OF CARBON IN PHOTOSYNTHESIS

VIII. THE RÔLE OF MALIC ACID*

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Exposure of various plants to C^{14}-labeled carbon dioxide under a number of different conditions both in the light and in the dark, followed by killing the plants and analysis of the labeled compounds formed by the plants, has led to the following experimental results (1).[1]

1. When the length of exposure of an actively photosynthesizing plant to labeled carbon dioxide in the light was shortened until nearly all the labeled carbon fixed by the plant was found in one compound, that compound was found to be phosphoglyceric acid (2). For example, when the green alga *Scenedesmus* was allowed to photosynthesize 5 seconds with labeled carbon dioxide and then killed, analysis showed that, of the total activity fixed and stable under the conditions of chromatographic analysis employed, 87 per cent was incorporated in phosphoglyceric acid, 10 per cent in phosphoenolpyruvic acid, and 3 per cent in malic acid.

2. As longer exposures to labeled carbon dioxide in the light are permitted (15 to 60 seconds), radioactivity is found not only in the above compounds, but also in aspartic acid, alanine, serine, glycine, glycolic acid, hexose diphosphate, triose phosphates, sucrose, and several as yet unidentified phosphorus-containing compounds (3, 4).

3. After still longer periods of exposure a large number of additional compounds are found to be labeled. These include succinic, fumaric, citric, and glutamic acids, glucose, fructose, and a number of amino acids (3-5).

4. When plants were exposed to $C^{14}O_2$ immediately following a period of illumination in the absence of carbon dioxide, the labeled products were found to be nearly the same as in the short exposures (15 to 60 seconds) in the light, although the proportion of radiocarbon found in malic acid, aspartic acid, and alanine to the total radiocarbon fixed was somewhat greater (5).

5. When the exposure to radioactive carbon dioxide in the dark did not

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[1] See Calvin and Benson (2) for a general discussion of previous experimental results and conclusions derived therefrom.
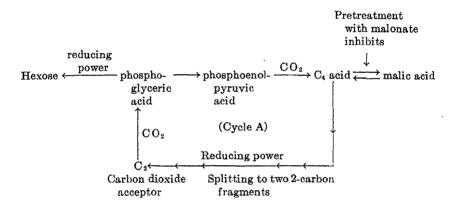
follow soon after a period of illumination, the labeled products (95 per cent of total) were malic, succinic, fumaric, citric, glutamic, and aspartic acids, and alanine (5).

6. Degradation of hexose formed during short periods of photosynthesis with labeled carbon dioxide revealed that the highest percentages of labeled carbon were in the 3 and 4 positions, the next highest in the 2 and 5 positions, and the least in the 1 and 6 positions. In some cases labeling of the 1 and 6 positions was found equal to that in the 2 and 5 positions (6). Degradation of phosphoglyceric acid (by methods described below) and of alanine demonstrated that the greatest labeling was in the carboxyl carbon.

In an attempt to correlate and explain these experimental results the following proposals have been made.

A. Most of the labeled products formed in the short periods of light or following preillumination are formed not at all or much more slowly in the dark without preillumination. Such compounds are considered to be primary (not dependent upon the prior formation of sugars) products of photosynthesis Into this classification fall the compounds listed under 1 and 2 above (2).

B. Those compounds found to be labeled in the dark non-preillumination experiments are formed at no appreciably greater rate in the light (with the exceptions of malic and aspartic acids and alanine); hence, such compounds (listed under (5) above) are considered to be formed by exchange in the reversible respiration reactions (5).

C. Similarity of products from light experiments and dark preillumination experiments indicates that the reactions involving carbon dioxide reductions are "dark reactions" and that reducing power, generated in the light, has a half life of several minutes (5, 7, 8).

D. It is proposed that phosphoglyceric acid is formed by the carboxylation of a 2-carbon compound and that this reaction is involved in photosynthetic carbon dioxide assimilation. This is suggested by the fact that phosphoglyceric acid is the first isolable compound and that its label first appears in the carboxyl group (2).

E. Part of the phosphoglyceric acid is believed to be reduced through the reversible reactions of glycolysis to give triose ˙phosphates, hexose phosphates, and sucrose (2).

F. Another part of the phosphoglyceric acid is believed to give phosphoenolpyruvic acid which could then be carboxylated as in the Wood-Werkman reaction (9) to give oxalacetic acid from which in turn malic and aspartic acids may arise. The enzyme system for such a carboxylation has been found in higher plants by Vennesland et al. (10).

G. There remains the necessity for continuously generating the 2-car-

bon compound which, according to the above proposals, is carboxylated to give phosphoglyceric acid. One possible way in which a C_2 compound might be formed would be a C_1 to C_1 condensation. This possibility is being investigated but is considered somewhat unlikely for several reasons. First, very little radioactivity has been found in 2-carbon compounds in the very short term experiments. Secondly, the phosphoglyceric acid is predominantly carboxyl-labeled (95 per cent of the total label of the molecule in 5 seconds light with *Scenedesmus*). Furthermore, only extremely small amounts of radiocarbon have been found in formaldehyde and formic acid after 2 minutes in the light. The absence of labeled, reduced C_1 compounds, together with the improbability of a direct coupling of 2 carbon dioxide molecules and the absence of any labeled oxalic acid in these experiments, argues against the C_1 to C_1 condensation.

The other alternative for the formation of a 2-carbon compound is for a 2-carbon fragment to be split from a larger molecule. The only larger molecules found to be labeled in the very short term photosynthesis experiments were the 3- and 4-carbon acids, phosphoglyceric, phosphoenolpyruvic acid, and malic acid, while at the same time a small but significant labeling of the α- and β-carbons of phosphoglyceric acid was found. Since the sum of the radioactivity found in the 3- and 4-carbon compounds in such short term experiments was equal within experimental error to the total non-volatile activity fixed during the experiment, it appears that all appreciably labeled compounds were detected by the methods of analysis employed. The splitting of a 3-carbon compound would result in either a profitless decarboxylation or in the formation of formaldehyde or formic acid, neither of which has been found to be labeled significantly even in longer experiments. Consequently, the most likely

regenerative mechanism would appear to be the splitting of a 4-carbon, dicarboxylic acid (cf. (F) above) to give 2 molecules of 2 carbons each which would be converted to the 2-carbon carbon dioxide acceptor. Thus, there would be a regenerative cycle consisting of C_1 to C_2 addition, C_1 to C_3 addition, splitting of a C_4 compound to two C_2 compounds, and reduction of the C_2 compound. This proposed cycle will be designated as Cycle A throughout this paper. Thus far, no experimental evidence has been obtained which would contradict the existence of the proposed Cycle A.

In attempting to find evidence for or against this cycle several dicarboxylic acids have been considered as possible intermediates in the Cycle A. Succinic and fumaric acids, tentatively suggested in earlier papers, appear more likely to be respiration intermediates than photosynthetic intermediates according to the reasoning in section (B). However, malic acid, because of its rapid labeling in the light, seemed a possible intermediate in the proposed cycle.

In order to ascertain whether malic acid might be such an intermediate, an attempt was made to inhibit its formation during short periods of photosynthesis.

In addition to the well known inhibition of succinic dehydrogenase by malonic acid, it has been reported that malonic acid inhibits the reduction of oxalacetic acid to malic acid (or the formation of malic acid from pyruvic acid and carbon dioxide) in animal tissues (11, 12). If the formation of malic acid could be appreciably inhibited and if, at the same time, some way could be found to measure the operation of the proposed cycle, then the participation of malic acid in the cycle could be tested.

Regardless of whether or not the proposed Cycle A exists, if the proposals in (D) and (E) are correct, then in order to be a photosynthetic intermediate between carbon dioxide and carbohydrate malic acid would have to be a precursor to the 2-carbon carbon dioxide acceptor and consequently a precursor to the α- and β-carbon atoms of phosphoglyceric acid. Therefore, by degrading phosphoglyceric acid and measuring the labeling of its α- and β-carbon atoms both in experiments in which labeled malic acid is formed without inhibition and in experiments in which the formation of labeled malic acid is inhibited it should be possible to determine whether malic acid is an intermediate in photosynthesis.

Methods and Material

In each pair of experiments a 1 day growth from 1 liter of a continuous culture of *Scenedesmus* (6) yielded 2.0 gm. of wet packed cells. 1 gm. of cells was suspended in phosphate buffer (0.007 M, pH 4.3) and the

other in phosphate buffer and 0.05 M sodium malonate[2] (pH 4.3). After various periods in the dark (Column A, Table I) the cells were centrifuged from the buffers, resuspended in 70 ml. of malonate-free phosphate buffer, and illuminated in a thin water-jacketed vessel. The removal of the cells from the malonate buffer prior to the exposure to labeled carbon dioxide was necessitated by the procedure for analyzing the cell constitu-

TABLE I

Effect of Pretreatment of Algal Suspensions with Malonate

Experiment No.	Time in dark	4 per cent CO₂ 96 per cent air and light	1 per cent CO₂ air and light	Air and light	C¹⁴O₂ and light	Total radioactivity fixed	Per cent of fixed radioactivity		Per cent inhibition of malic acid	Distribution of radioactivity in glyceric acid, per cent of starting activity fixed as PGA				Per cent of total radioactivity fixed in aspartic acid
							PGA*	Malic acid		Carboxyl	α	β	Total	
	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)	(K)	(L)	(M)	(N)
	min.	min.	min.	min.	sec.	μc.								
M-1	150	30	10	0	60	5.0	19	2.41	70	50	25	30	105	1.50
B-1	210	30	10	0	60	7.7	17	8.14		55	25	27	107	1.68
M-2	255	30	15	0	60	7.7	22	1.64	66	41	25	29	95	2.13
B-2	315	30	15	0	60	9.0	28	4.86		44	25	31	99	2.19
M-3	180	40	0	0	30	1.6	30	0.42	83	73	12	15	100	
B-3	240	40	0	0	30	2.4	35	2.48		81	7	10	98	
M-4	210	30	0	10	90	14.0		0.45	97	50	50			3.86
										~10†	~10†			
B-4	270	30	0	10	90	15.9		13.90		55	45			5.93
										~10†	~10†			

Light intensity = 2500 foot-candles for Experiments M-1 to B-3, 10,000 foot-candles for Experiments M-4 and B-4. Temperature 22°. Experiments M-1, M-2, M-3, and M-4, malonate inhibition; Experiments B-1, B-2, B-3, and B-4, the controls (no malonate).

* Phosphoglyceric acid.

† In Experiments M-4 and B-4 alanine rather than glyceric acid was degraded.

ents after killing. Had the sodium malonate been present when the cell extract was concentrated and applied to the paper the chromatogram would have been overloaded by the large quantity of sodium malonate. Streams of air with different percentages of carbon dioxide were passed through the cell suspension for 30 to 45 minutes (conditions as in Columns B, C, D, Table I, performed in that order) and then the radioactive sodium bicarbonate solution (100 μc., 0.02 mM) was added, the flasks

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[2] Identical experiments with radioactive malonate demonstrated that the concentration of malonate within the cells (or adsorbed on the cell surface) was 0.015 M at the time of the analysis of photosynthetic products.

stoppered, and the cells allowed to photosynthesize for 30 to 90 seconds (Column E) before being killed in boiling ethanol. The alcohol extract was then analyzed by paper chromatography and radioautography (4). The percentages of the total fixed activity found in malic acid are given in Column H, Table I.

The phosphoglyceric acid obtained from the cell extract was hydrolyzed in acid and the hydrolysate rechromatographed to give a single spot of glyceric acid. The glyceric acid was eluted from the paper and recrystallized twice with unlabeled calcium glycerate. The specific activity of the resulting labeled calcium glycerate was determined and the following degradation carried out.

50 mg. of the labeled calcium glycerate (0.4 mM) were placed in a flask with 0.80 ml. of 1.0 N periodic acid. After 2 hours, the solution was made slightly alkaline and the volatile contents, including formaldehyde, were distilled *in vacuo* into a solution of dimethyldihydroresorcinol from which the dimethone compound of formaldehyde was isolated and recrystallized.

To the non-volatile residue of sodium glyoxylate, 5 ml. of 1.0 N periodic acid were added, and after 24 hours the volatile contents were distilled into a carbonate-free sodium hydroxide solution. Barium chloride solution was then added, the barium carbonate precipitate centrifuged, washed, and dried, and the supernatant solution was acidified and steam-distilled to collect the formic acid. The steam distillate was neutralized with barium hydroxide and concentrated to dryness. The barium formate was recrystallized from water and alcohol. The specific activities of the barium carbonate, barium formate, and dimethone compound were determined, and, with the theoretical yields, gave the total radioactivities of the carboxyl, α- and β-carbons (expressed as percentages of starting radioactivity in Columns J, K, and L of Table I).

The results are given in Table I. It is seen that pretreatment with malonate caused an appreciable decrease in the radioactivity incorporated in malic acid (66 to 97 per cent) and a much smaller decrease in total activity fixed (12 to 35 per cent). The labeling of aspartic acid was not appreciably decreased in two experiments and was decreased 35 per cent in a third experiment. The per cent of the total labeling of the phosphoglyceric acid molecule found in the α- and β-carbons was as great or greater in the experiments in which malic labeling was decreased by malonate pretreatment of the cells as in the experiments in which no malonate pretreatment was employed. No labeled succinic or fumaric acid was detected in any of the experiments.

These results indicate that the malonate pretreatment may have decreased slightly the rate of the carboxylation of C_3 compound to give C_4 dicarboxylic acid, as may be evidenced by the decrease in total radiocarbon fixed and by the decrease in aspartic acid labeling in one case, but

that the formation of malic acid is much more strongly inhibited by the malonate pretreatment.

The undiminished labeling of the α- and β-carbons of phosphoglyceric acid at the same time that malic acid labeling is greatly decreased indicates that malic acid is not a precursor of the α- and β-carbon atoms of phosphoglyceric acid. If the proposal is correct that phosphoglyceric acid is an intermediate in photosynthesis and is reduced through reversible glycolysis reactions to carbohydrate, then malic acid cannot be a photosynthetic intermediate.

The absence of labeled succinic and fumaric acid in the above experiment indicates that these two acids are not precursors of the α- and β-carbon atoms of phosphoglyceric acid and hence by the above reasoning not photosynthetic intermediates.

SUMMARY

Pretreatment of algal suspensions with malonate, followed by short periods of photosynthesis with radioactive carbon dioxide, has been found to inhibit the formation of labeled malic acid.

Degradation of phosphoglyceric acid formed at the same time shows no decrease in per cent labeling of the α- and β-carbon atoms over that obtained with cells not pretreated with malonate.

These results suggest that malic acid is not a precursor of the α- and β-carbons of phosphoglyceric acid in photosynthesis.

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RAT LIVER FRUCTOKINASE*

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The difference in the rates of anaerobic glycolysis between tumor tissue and normal tissue led us to believe that a study of hexokinase activity in tissues from leucemic animals would prove of interest. We proceeded, therefore, to study sugar phosphorylation in normal rat liver homogenates in an attempt to standardize the procedures. Preliminary experiments confirmed reports that glucose was not readily phosphorylated in liver homogenates, and there seemed to be need of further investigation of liver hexokinase systems.

The synthesis of glycogen from glucose in perfused liver and in liver *slices* from several species has been studied by several groups (1–10) with somewhat varying results. Buchanan *et al.* (11) have recently emphasized the importance of potassium ions and of a very high level of glucose (1 per cent) in the medium bathing the slices. Colowick, Welch, and Cori (12), Colowick, Kalckar, and Cori (13), and Price, Cori, and Colowick (14) have reported the oxidation and phosphorylation of glucose in cell-free rat and rabbit liver extracts. However, liver homogenates or extracts have been found by various investigators (4, 15–17) to be unable to phosphorylate glucose effectively. Indeed, in several studies involving glucose phosphorylation in liver systems, yeast hexokinase has been added (18–20). In an attempt to find favorable conditions for glucokinase activity in liver homogenates, we studied this reaction under many different conditions. Since only relatively small amounts of glucose were taken up despite numerous variations in the procedure, our attention was directed to the phosphorylation of fructose (21–30). Fructose was found to be phosphorylated readily in a system containing adenosine triphosphate (ATP), phosphate buffer, glutamate or α-ketoglutarate, $MgCl_2$, and a slightly hypertonic liver homogenate (31).

This report describes an investigation of fructokinase activity in liver homogenates and a study of some of the problems that arose.

Adult albino rats of both sexes, which had been fasted for 16 to 24 hours, were stunned, decapitated, and exsanguinated. The liver was removed rapidly, placed on cracked ice for several minutes, and appropriate portions (1 to 2 gm.) weighed directly into a homogenizing tube. 4 ml. of ice-cold potassium phosphate buffer (pH 7.5, 0.15 M) were added per gm. of liver and the mixture was homogenized for 2 to 4 minutes in a Potter-Elvehjem glass homogenizer (32). The resulting homogenate was strained through cheese-cloth. In early experiments this "20 per cent homogenate" was diluted with phosphate buffer to give a 10 per cent homogenate.

Most systems were incubated for 30 minutes at 37° in a conventional Warburg apparatus after a 5 minute equilibration period. All reactants were added to the main compartment of the flask which was kept at room temperature, or in a few cases at 0°, before being placed in the bath. When fluoride was added, it was placed in the main compartment after all other components including the homogenate had been added. If ATP, fructose, and α-ketoglutarate were kept in the side arm during the equilibration period, fructose phosphorylation was decreased. The components of the "best" system for fructose phosphorylation are as follows: 0.2 ml. of 0.08 M $MgCl_2 \cdot 6$ H_2O, 0.2 ml. of 0.154 M $KH_2PO_4 \cdot K_2HPO_4$ (pH 7.5), 0.2 ml. of 0.5 M sodium or potassium L-glutamate or 0.2 ml. of 0.1 M potassium α-ketoglutarate, 0.2 ml. of 0.05 M adenosine triphosphate ($Na_4ATP \cdot 3H_2O$), 0.2 ml. of 0.056 M (1 per cent) fructose, 0.4 ml. of 20 per cent liver homogenate, and water to make a final volume of 2 ml. All components were adjusted to pH 7.5. 0.1 ml. of 20 per cent KOH was added to the center well when oxygen uptake was recorded. Any deviations from the procedures above are noted in the legends of the tables.

Fructose disappearance was measured by the Nelson procedure for reducing sugar (33) and according to the method of Roe (34). Slight differences in the reducing power of fructose and glucose in the Nelson determination were neglected. After incubation 1 ml. of the reaction mixture was pipetted into 2 ml. of $ZnSO_4$ solution. Next were added 2 ml. of $Ba(OH)_2$ solution and water to make a total volume of 10 ml. with a final pH of 7.0 to 7.6. Control experiments showed that hexose phosphates were quantitatively precipitated by this method. The precipitate was removed by filtration and aliquots of the filtrate (usually 1 ml.) were used for analysis. The reducing or fructose values thus obtained were subtracted from the amount of fructose added. Added fructose was determined by running control flasks to which was added everything but the fructose. After incubation 1 ml. of the control reaction mixture

was added to 2 ml. of ZnSO₄ solution, followed by 0.1 ml. of 1 per cent fructose solution. The deproteinization was then completed as described above. The same procedure was employed to calculate reducing values with other substrates.

Brain hexokinase was prepared according to the method of Colowick, Cori, and Slein (35), except that 6 volumes of water were used to obtain the extract from the powder.

L-Glutamic acid was obtained from Merck and Company and D-fructose from the Pfanstiehl Chemical Company. The barium salt of fructose diphosphoric acid was the product of the Schwarz Laboratories. It was recrystallized several times according to the method of Neuberg *et al.* (36). The sodium salt of fructose diphosphoric acid was prepared from the barium salt by dissolving the latter in 0.1 N HCl, adding saturated Na₂SO₄ in small excess, centrifuging, and neutralizing the supernatant liquid. Glucose-1-phosphate, α-ketoglutaric acid, fructose-6-phosphate, and barium ATP were prepared and recrystallized several times according to the methods outlined in Umbreit *et al.* (37). Sodium ATP was the Rohm and Haas product. We wish to thank Dr. J. N. Williams, Jr., of the University of Wisconsin, for a sample of glucose-6-phosphate.

Fructose-6-phosphate and fructofuranose diphosphate (FDP) were determined according to the method indicated in Umbreit *et al.* (37) (Procedure A). The water-insoluble barium fraction was tested for FDP by the Roe method. The water-soluble, alcohol-insoluble barium fraction was tested for fructose-6-phosphate by the Roe method. Each time unknown solutions were analyzed for fructose, a standard curve was prepared. Since the color was found to be unstable, the Evelyn colorimeter tubes were placed in ice water after the 8 minute heating period at 80° and the color read 10 minutes later. Fructose-6-phosphate and FDP concentrations were obtained from a standard fructose curve by multiplying by the appropriate factors as directed in Umbreit *et al.* (37). Small amounts of added fructose were found not to interfere with the analysis of fructose-6-phosphate and FDP. When given amounts of FDP were added to test samples, recoveries were only 60 to 90 per cent efficient; fructose-6-phosphate recoveries were more satisfactory.

Results

Components of the rat liver fructokinase system and optimum concentrations are presented in Table I. Added MgCl₂ and ATP are essential for the phosphorylation of fructose. In the absence of added α-ketoglutarate or glutamate there is less than one-half of the maximum fructose uptake. Glutamate and α-ketoglutarate were both effective in increasing fructose phosphorylation, although only about one-third as

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TABLE I

Components of Rat Liver Fructokinase System

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Data from separate experiments. α-Ketoglutarate (final concentration, 0.01 M) or glutamate (final concentration, 0.05 M) has been used interchangeably in these experiments, except where otherwise indicated. 0.5 ml. of 10 per cent liver homogenate was added per flask. All flasks contained NaF (final concentration, 0.05 M).

Fructose disappearance*									
ATP†		MgCl₂		Dicarboxylic acid				Phosphate	
				α-Ketoglutarate		Glutamate			
M	mg.‡	M	mg.‡	M	mg.‡	M	mg.‡	M	mg.‡
0	0	0	0.14	0	0.66	0	0.66	0§	0.56
0.001	1.23	0.008	1.28	0.002	1.28	0.002	1.12	0.04	1.62
0.003	1.32			0.004	1.58	0.004	1.20		
0.005	1.32			0.01	1.70	0.01	1.40		
0.0075	1.23			0.025	1.64	0.025	1.60		
0.01	1.26			0.05	1.52	0.05	1.60		

* Fructose added per flask, 2 mg.

† The barium salt of ATP was used. It was converted to the sodium salt prior to its addition to the system.

‡ Fructose disappearance.

§ Potassium diethyl barbiturate (0.037 M, pH 7.5) was used as buffer and homogenizing medium.

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TABLE II

Comparison of Effect of α-Ketoglutarate and Other Dicarboxylic Acids on Fructose Phosphorylation in Rat Liver Homogenates

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All flasks contained 0.2 ml. of 0.154 M potassium phosphate buffer, 0.2 ml. of 0.08 M MgCl₂, 0.1 ml. of 0.1 M sodium ATP, 0.2 ml. of 0.056 M fructose, 0.5 ml. of 10 per cent liver homogenate, 0.2 ml. of 0.5 M NaF, and water to bring the final volume to 2 ml. 0.2 ml. of 0.1 M potassium α-ketoglutarate, succinate, fumarate, malate, or oxalacetate was added as indicated.

Dicarboxylic acid added	Fructose disappearance
	mg.
None	0.70
α-Ketoglutarate	1.28
Oxalacetate	0.74
Fumarate	0.80
Succinate	0.82
Malate	0.90
α-Ketoglutarate + malonate	0.76*

* 0.1 ml. of 0.75 M sodium malonate was added.

much of the latter is required.   This is in accordance with the idea that glutamate has first to be converted to α-ketoglutarate.

If dicarboxylic acids other than α-ketoglutarate or glutamate are added to the system, fructose phosphorylation is increased to a smaller extent (Table II).   The addition of malonate to a complete system results in fructose uptake comparable to that obtained in the absence of α-ketoglutarate.   If malonate is assumed to interfere with the reaction, α-ketoglutarate → succinate (38), it would appear that this step is involved in increasing fructose uptake in the type of liver homogenate system used in these studies.

TABLE III

Effect of Homogenizing Medium on Phosphorylation of Fructose in Rat Liver Homogenates

Homogenization was carried out in ice-cold redistilled water in the case of "water homogenates," in 0.154 M potassium phosphate (pH 7.5) in the case of "phosphate homogenates," and in 0.1 M potassium diethyl barbiturate (pH 7.5) for "veronal homogenates."   The components of the reaction mixture were as follows: 0.2 ml. of 0.08 M MgCl₂, 0.2 ml. of 0.1 M potassium α-ketoglutarate, 0.1 ml. of 0.112 M fructose, 0.1 ml. of 0.1 M sodium ATP, 0.2 ml. of 0.5 M NaF, 0.2 ml. of 0.154 M potassium phosphate buffer (pH 7.5), 0.5 ml. of 10 per cent liver homogenate, and water to make a total flask volume of 2 ml.   To flasks containing water homogenates additional phosphate was added to bring the final phosphate concentration to 0.04 M.

Homogenizing medium	Fructose disappearance
	mg.
Water	0.24
"	0.28
Phosphate	1.62
"	1.54
Veronal	0.90

If water is used as the homogenization medium, almost complete loss of activity results (Table III).   Homogenization in veronal buffer also diminishes fructokinase activity.   If phosphate is entirely omitted from the system, fructose phosphorylation is also decreased (Table I).   The fact that water homogenization destroys the activity of the system suggests that some sort of "dissociable factors" seem to be involved.   Active homogenates lose their activity rather rapidly (several hours) when kept either at 0° or at room temperature.

In early experiments oxygen uptake was recorded.   The results showed that both in the presence and absence of added glutamate, fructose uptake was not obviously associated with increased oxygen consumption,

The addition of diphosphopyridine nucleotide (DPN), cytochrome c, or FDP did not change these results. It was, therefore, of interest to investigate the fate of fructose in the system. Analyses were performed on the mixture after incubation, and the results explained the lack of oxygen uptake during fructose phosphorylation. As shown in Table IV, after $\frac{1}{2}$ hour of incubation, three-fourths of the added fructose is recovered as "apparent fructose-6-phosphate," one-tenth as FDP, and a small fraction reappears as glucose (calculated from the difference in values obtained by the Nelson and Roe procedures). After a 2 hour incubation period an increase in reducing power results. If the analysis is

TABLE IV

End-Products of Fructose Phosphorylation in Rat Liver Homogenates

The components of the reaction mixture and the methods of analysis are described in the text.

Experiment No.	Incubation time	Fructose recovered as				Fructose added initially
		"Apparent fructose-6-phosphate"	FDP	Glucose	Fructose	
	hrs.	*mg.**	*mg.**	*mg.*	*mg.*	*mg.*
73	$\frac{1}{2}$	1.35	0.26	0.20	0.38	2.06
77	$\frac{1}{2}$	1.55	0.11	0.13	0.56	1.88
78	$\frac{1}{2}$ ·	1.68	0.19	0.17	0.21	1.94
78	$\frac{1}{2}$			0.38†	0.54	1.94
73	2	0.92	0.17	0.84	0.13	2.06
74	2			0.54	0.18	2.12
75	2			1.24	0.07	1.98
76	2			1.69	0.13	1.98

* Expressed as mg. of fructose.
† Fructose-6-phosphate added to make a final concentration of 0.00056 M.

carried out by the Nelson procedure only, this appears to represent a decrease in fructose uptake. However, if both Roe and Nelson methods are used, the increase in reducing power seems to be due to a breakdown of "apparent fructose-6-phosphate" to glucose. If this assumption is correct, fructose-6-phosphate itself should behave in a similar manner. This was found not to be the case. As indicated in Table V, over half of the added fructose-6-phosphate is converted to glucose in $\frac{1}{2}$ hour. But in Table IV it was shown that three-fourths of the fructose added was recovered in the water-soluble, alcohol-insoluble barium fraction, whose only "fructose-reacting" constituent is supposed to be fructose-6-phosphate (37). It would appear either that the "fructose-6-phosphate" is not fructose-6-phosphate or that the fructose-6-phosphate prepared con-

tains a catalytic intermediate which speeds the conversion of fructose-6-phosphate to glucose. To test the latter idea a small amount of fructose-6-phosphate was added to a complete system (Table IV). The conversion of fructose to glucose was not increased significantly. Similar results were obtained by adding small amounts of FDP. Another observation leads us to question the fructose-6-phosphate values. If all fractions are analyzed and the results totaled, fructose recovery is over 100 per cent despite the fact that the methods of analysis are not 100 per cent efficient in our hands and the fact that when FDP is added to the system, one-half of it disappears. But if "apparent fructose-6-phosphate" is not fructose-6-phosphate, the factor (37) used in calculating

TABLE V

Fate of Fructose-6-phosphate in Rat Liver Homogenates

The components of the reaction mixture and the methods of analysis are described in the text.

| Experiment No. | Incubation time | Fructose-6-phosphate recovered as | | | Fructose-6-phosphate added initially |
		Glucose	FDP	Fructose-6-phosphate	
	hrs.	*mg.**	*mg.**	*mg.**	*mg.**
75	½	1.12			2.0
76	½	1.43			2.0
77	½	0.89	0.29	0.20	1.89
78	½	0.88	0.17	0.13	1.89
75	2	1.35			2.0
76	2	1.80			2.0

* Expressed as mg. of fructose.

the fructose-6-phosphate values might be in error and thus account for the extra fructose recovered. Further work is required to clarify this point.

The majority of experiments reported in this paper were performed on rat liver homogenates. The average and the range of the values obtained are recorded in Table VI. Mouse and guinea pig liver systems appear to have a lesser capacity to phosphorylate fructose. It is likely that optimum conditions for various species differ. Liver homogenates of fed or fasted rabbits showed an inability to phosphorylate fructose under the conditions described. None of the liver homogenates of the different species showed appreciable glucokinase or galactokinase activity.

This raises the question as to why glucose is not taken up in this system to any appreciable extent. There are at least three possible answers: experimental conditions are not favorable; glucose is phosphorylated in

the system, but the glucose-6-phosphate is just as rapidly hydrolyzed enzymatically; or active glucokinase is not present as such.

On the assumption that the system described in this paper was not favorable for glucokinase activity, numerous variations were introduced. We were unable to demonstrate appreciable phosphorylation of glucose in rat liver homogenates by adding DPN and nicotinamide, cytochrome c, FDP, phosphocreatine, fumarate, $MnCl_2$ instead of $MgCl_2$, myokinase, adenylic acid, insulin, adrenal cortical extract, malonate, or Ca^{++} in various combinations. Substitution of bicarbonate for phosphate did not increase glucose uptake.

TABLE VI

Hexokinase Activity in Liver Homogenates from Rats and Other Species

The components of the reaction mixture are described in the text. Equal amounts (2 mg.) of fructose, glucose, or galactose were used. All animals were fasted for 24 hours, unless otherwise indicated.

Species	Sugar disappearance		
	Fructose	Glucose	Galactose
	mg.	*mg.*	*mg.*
Rat..........................	1.47*	0.13†	−0.20
Mouse‡.......................	0.80	−0.01	
" (Swiss)................	0.60		
" (C58)..................	0.58	0.04	
Guinea Pig...................	0.76	0.38	
Rabbit.......................	0.16	0.02	0.14
" (fed).................	−0.08	−0.09	0.33

* Average of thirty-eight experiments; range, 1.10 to 1.83 mg.

† Range of numerous experiments, 0 to 0.28 mg. (with and without NaF).

‡ Insulin-resistant mouse (see Chase, H. M., *et al.* (40)).

Phosphatase inhibitors such as cysteine, thioglycolate, $LiSO_4$, α-glycerophosphate, and NaF were ineffective in bringing about a significant net disappearance of glucose. Fractionation of the homogenate and the use of either supernatant fluid, particles, or washed particles did not result in glucose uptake. Neither did incubation of the liver homogenate anaerobically or in an atmosphere of oxygen increase glucose consumption.

Since a great number of variations of the experimental conditions did not result in appreciable glucokinase activity, another experiment was devised to demonstrate whether or not conditions were favorable for glucose uptake in the fructokinase system. A crude brain hexokinase preparation (35) was added to the system and the result was a glucose uptake quite comparable to that obtained with fructose (Table VII). This

seems to indicate that conditions in the system are favorable for gluco-kinase activity. The fact that more glucose uptake occurs in the presence of both brain extract and liver homogenate than with brain extract alone is of interest. This effect is abolished by boiling the liver homogenate. Another interesting point is that if both brain extract and liver

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TABLE VII

Sugar Disappearance in Rat Liver Homogenates in Presence of Crude Preparations of Brain Hexokinase

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In Experiments 28 and 30 all flasks contained 0.2 ml. of 0.5 M sodium L-glutamate, 0.2 ml. of 0.08 M MgCl$_2$, 0.2 ml. of 0.05 M sodium ATP, 0.2 ml. of 0.154 M potassium phosphate buffer (pH 7.5), and 0.2 ml. of 0.5 M NaF. Other flask additions as specified in the table: 0.5 ml. of brain extract (B.), 0.4 ml. of 20 per cent liver homogenate (L.), 0.2 ml. of 0.056 M fructose (F.) or glucose (G.), and water to make a final volume of 2 ml. To flasks not containing liver homogenate or brain extract additional 0.154 M phosphate was added. In Experiments 92 and 93 the flask contents were as above except that 0.5 ml. of 10 per cent liver homogenate, 0.2 ml. of 0.1 M sodium α-ketoglutarate (instead of glutamate), and a different crude brain hexokinase preparation were used. In the table BB. denotes heat-inactivated brain extract and BL. denotes heat-inactivated liver homogenate.

Experiment No.	Sugar	Sugar disappearance				
		B.	L.	B. + L.	BB. + L.	B. + BL.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*
28	F.	0.59	1.55	1.49	0.90	
28	"	0.26	1.61	1.44		
28	" *	0.33	0.47	1.19		
28	G.	0.50	0.23	1.14	−0.20	
30	F.	0.52	1.65	1.94	1.48	
30	G.	0.27	0.17	1.02		
92	F.		1.48			
92	G.	0.87	0.10	1.92	0.08	0.62
93	F.		1.42			
93	G.	0.88	−0.08	1.56		
93	" *			1.40		

* These flasks contained no added glutamate or α-ketoglutarate.

homogenate are added, glutamate or α-ketoglutarate can be omitted from the system.

Other experiments seem to demonstrate not only that conditions are favorable for glucose phosphorylation in the system, but also that phosphatase activity in liver homogenates is not sufficiently strong to explain the apparent lack of glucokinase activity. This is shown by the data reported in Table VIII. Phosphatase activity is not very appreciable in

the system during a half hour incubation period in the presence of NaF. (The reducing values were obtained with the Nelson procedure and therefore, labeled "apparent glucose.")

Experiments with liver slices yielded similar results. Table IX shows that, if glucose is added in concentrations of 1 mg. per ml., it is not taken up to any appreciable extent by rat liver slices under the conditions of these experiments. The extent of fructokinase activity, on the other

TABLE VIII

Phosphatase Activity in Rat Liver Slices and Homogenates

The reaction mixture contained 0.2 ml. of 0.5 M sodium L-glutamate, 0.2 ml. of 0.154 M potassium phosphate buffer (pH 7.5), 0.2 ml. of 0.08 M $MgCl_2$, 0.2 ml. of 0.05 M sodium ATP, 0.2 ml. of 0.5 M NaF, 0.4 ml. of 20 per cent liver homogenate or liver slices (approximately 0.16 gm. wet weight), and water to bring the final flask volume to 2 ml. Glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, or fructose-1,6-diphosphate was added as indicated so as to be present in 0.0056 M final concentration. Liver slices were prepared free-hand and with a Stadie-Riggs microtome.

Substrate added	Increase in reducing value of "apparent glucose"	
	Homogenate	Slices
	mg.	*mg.*
Glucose-1-phosphate....................	0.31 (4)*	0.30 (4)
"	0.50†	
Glucose-6-phosphate....................	0.43 (3)	0.45 (4)
"	0.66†	
Fructose-6-phosphate..................	0.51 (6)	0.20 (3)
"	0.74†	
"	0.81†	
Fructose diphosphate..................	0.26 (4)	

* The numbers in parentheses denote the number of experiments performed. The ranges in mg. are as follows: glucose-1-phosphate, 0.22 to 0.36; glucose-6-phosphate, 0.38 to 0.46; fructose-6-phosphate, 0.44 to 0.61; fructose diphosphate, 0.20 to 0.32.

† NaF omitted.

hand, is about the same in liver slices as in homogenates. If 5 mg. per ml. of glucose are added to rat liver slices, it appears as if 0.8 mg. of glucose is taken up by the slices. Therefore, a number of experiments were performed in which the slices were homogenized after incubation to release any "free" sugar. When glucose uptake was calculated on the basis of this procedure, only about 0.2 to 0.3 mg. was found to disappear. But fructose uptake was not affected.

These experiments lead us to believe that glucokinase activity is very difficult to demonstrate compared to fructokinase activity in liver slices

and homogenates. The question arises as to whether these results obtained on isolated tissue preparations are of physiological significance. Bouckaert and de Duve (39) have advanced the hypothesis that glucose phosphorylation takes place through the adsorption of glucose on "hexokinase" and the subsequent activation of the adsorbed glucose, possibly by conversion to fructofuranose. The activated glucose is only then phosphorylated by ATP. If fructofuranose were an intermediate in the

TABLE IX

Hexokinase Activity in Rat Liver Slices

The components of the reaction mixture were as follows: 0.2 ml. of 0.5 M sodium L-glutamate, 0.2 ml. of 0.08 M MgCl₂, 0.8 ml. of 0.154 M potassium phosphate buffer (pH 7.5), 0.2 ml. of 0.05 M sodium ATP, 0.2 ml. of 0.5 M NaF, and water to bring the final flask volume to 2 ml. Liver slices were prepared free-hand and with the Stadie-Riggs microtome. 150 to 200 mg. wet weight of slices per flask. Data from separate experiments.

No. of experiments	Sugar	Amount added	Sugar disappearance (average and range)
		mg.	*mg.*
6	Fructose	2	1.14 (0.98–1.28)
1	"	2	1.16*
1	"	2	0.50†
3	"	10	2.17 (2.10–2.30)
1	"	10	2.30‡
3	Glucose	2	0.12 (0.01–0.28)
3	"	10	0.80 (0.60–0.90)
3	"	10	0.27‡(0.20–0.30)

* NaF omitted.
† ATP omitted.
‡ After incubation the slices were subjected to homogenization and the homogenates were analyzed.

phosphorylation of glucose in the liver, the ready phosphorylation of fructose could be understood. Further experiments are needed either to prove or disprove this idea.

SUMMARY

1. Fructokinase and glucokinase activity have been studied in rat liver homogenates and slices. In the presence of ATP, MgCl₂, α-ketoglutarate or glutamate, and phosphate buffer, fructose phosphorylation occurred 10 times more readily than glucose phosphorylation. If water was used as the homogenizing medium instead of slightly hypertonic phosphate buffer, fructokinase activity was lost.

2. A preliminary study has been made of the fate of fructose and fructose-6-phosphate in the system.

3. Other species of animals showed liver fructokinase activity qualitatively similar to that obtained with the rat, with the exception of the rabbit which was found to be unable to phosphorylate fructose under the conditions of these experiments.

4. Glucose was taken up only to a small extent in liver slices and homogenates under many different experimental conditions. However, if a crude brain hexokinase preparation was added to liver homogenates, glucose phosphorylation proceeded rapidly. Phosphatase activity seems to be unable to account for the lack of glucose uptake in these systems.

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SELENATE INHIBITION STUDIES

IV. BIOCHEMICAL BASIS OF SELENATE TOXICITY IN YEAST*

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In a previous paper it was reported that DL-methionine was capable of partially reversing the toxic effect of selenate in yeast (1). A closer investigation revealed that only the L isomer of the amino acid was active, whereas the D form was without effect. These results appeared to indicate that the organism was enzymatically active in detoxifying selenium salts, a finding supported by the recent demonstration of a selenate-decomposing enzyme in mammalian tissue (2).

The present study was designed to investigate further the behavior of methionine in the detoxication process and gain general information on the mode of action of selenate in yeast. Several plausible explanations have suggested themselves, each of which has a precedent in other biochemical phenomena associated with methionine. These are treated separately below.

EXPERIMENTAL

The same general procedure as that described previously (1) was followed in these experiments. The same organism was used as in the previous studies, *Saccharomyces cerevisiae* (Fleischmann).

Oxidation of Methionine to Sulfate

Sulfate can counteract selenate inhibition in both yeast (3) and green plants (4, 5), and it is known that methionine may be oxidized to sulfate in humans (6), rabbits (7), dogs (8), green plants (9), and *Aspergillus niger* (10). It therefore appeared that, if yeast could be shown to oxidize methionine to sulfate, an explanation of the reversal process would be at hand.

The first experiment was designed to investigate the oxidative capacity

of "resting" yeast cells with respect to methionine. Three flasks, each containing 1 gm. of DL-methionine in volumes of 250 ml. of dilute phosphate buffer, pH 5.2, were inoculated with a heavy suspension of washed yeast cells (about 1 mg., wet weight, per flask). Controls containing no methionine were also prepared in triplicate. The flasks were then incubated at 30° for 24 hours with occasional shaking. The determination of sulfate was performed gravimetrically according to the method of Wood and Barren (11). With this procedure, no sulfate could be detected either

TABLE I

Sulfate-Free Medium Used in Oxidation Experiments

DL-Methionine...	6 gm.
Sucrose...	30 "
NH₄Cl..	4.5 gm.
KH₂PO₄..	2 gm.
CaCl₂·2H₂O...	0.4 gm.
MgCl₂..	0.49 gm.
Inositol..	4 mg.
Thiamine hydrochloride....................................	30 γ
Calcium pantothenate......................................	400 "
Pyridoxine...	30 "
Biotin...	0.4 γ
H₂O to pH 5.2..	1500 ml.

TABLE II

Oxygen Consumption of Yeast Cells in Presence and Absence of Methionine

Methionine added, γ......................	0*	300	600	900	600 (Side arm)
O₂ uptake, *c.mm*..................	19.6	18.9	20.7	20.9	18.9

* Contains only yeast cells and buffer.

in the presence or absence of methionine, nor could it be found in a medium containing actively growing yeast cells in which methionine was used as the sole sulfur source (Table I), either with or without added selenate (30 mg. of H_2SeO_4 per 250 ml.). However, control flasks containing, in addition to methionine, 11 mg. of sulfate in 250 ml. of medium also gave negative tests for sulfate, whereas similar uninoculated flasks were positive.

The possibility that only small amounts of sulfate were being formed was investigated by studying the O_2 uptake in Warburg respirometers. Washed cells (about 9 mg. of wet weight) were suspended in phosphate buffer at pH 5.2 in the presence and absence of methionine. The inner wells contained Whatman No. 40 filter paper impregnated with 20 per

:ent KOH to absorb CO_2. The total volume of the contents in the War-ourg flasks was 3.3 ml. These were then shaken for 3 hours at 30°. The thermobarometrically corrected oxygen consumption for this period (given in Table II) was essentially the same in all flasks, indicating that sulfate was not being formed under these conditions.

TABLE III

Effect of Sulfur Compounds in Enhancing Reversing Action of Methionine against Selenate

Added per 10 ml.

H_2SeO_4	Methionine	Cystine	Cysteine*	Glutathi-one	Thiamine*	Biotin*	Optical density†
mg.	mg.	mg.	mg.	mg.	γ	γ	
0	0	0	0	0	0	0	0.15
0	2	0	0	0	0	0	0.88
1	2	0	0	0	0	0	0.61
0	0	2	0	0	0	0	0.21
1	0	2	0	0	0	0	0.00
0	0	0	0	2	0	0	0.20
1	0	0	0	2	0	0	0.00
0	2	2	0	0	0	0	0.81
1	2	2	0	0	0	0	0.57
0	2	2	0	2	0	0	0.88
1	2	2	0	2	0	0	0.55
0	2	0	0	0	0	0	0.92
1	2	0	0	0	0	0	0.72
1	2	0	1	0	0	0	0.72
1	2	0	0	0	1	0	0.90
1	2	0	0	0	0	0.01	0.77
2	2	0	0	0	0	0	0.58
2	2	0	1	0	0	0	0.65
2	2	0	0	0	1	0	0.71
2	2	0	0	0	0	0.01	0.60
3	2	0	0	0	0	0	0.46
3	2	0	0	0	1	0	0.61

* Tubes containing cysteine, thiamine, and biotin were incubated for 52 hours. The others were incubated for 48 hours.

Growth and Reversal with Methionine and Other Sulfur Compounds

Methionine has consistently been found to reverse the toxic effect of selenate only partially (1). Experiments were therefore designed to test the effect of other sulfur compounds in enhancing the reversing power of methionine. As may be seen from Table III, glutathione, cystine, and biotin are without effect, but cysteine and thiamine are active in this re-

spect. Thiamine restores full growth at a level of 1 mg. of selenate per tube, but at higher levels of selenate the restorative effect is not complete, even at concentrations of 10 γ of thiamine per tube, nor with any combination of the other sulfur compounds tested (not shown in Table

TABLE IV

*Comparison of Yeast Growth in Sulfate and Methionine**

Experiment No.	Incubation	Optical density	
		Na₂SO₄·10H₂O	DL-Methionine
	hrs.		
1	43	0.72	0.70
2	48	0.96	0.88
3	67	1.25	1.25
4	36	0.69	0.68
5	120	0.99	0.98
6	120	1.02	1.01
7	120	0.98	1.01
8	43	1.08	1.00
9	43	0.98	0.98

* 2.5 mg. of $Na_2SO_4 \cdot 10H_2O$ and 2 mg. of DL-methionine per tube were used respectively. The assumption was made initially that only the L isomer of methionine could be utilized; thus these amounts were considered equivalent. It was found later that the D form was slightly active, but no corrections were made in the quantities used, in view of the equal growth observed with the two substances in the amounts listed above.

TABLE V

Influence of Thiamine on Yeast Growth in Presence of Sulfate and Methionine

Thiamine	Optical density	
	Na₂SO₄·10H₂O (2.5 mg.)	DL-Methionine (2 mg.)
γ		
0	0.51	0.53
0.01	0.56	0.52
0.05	0.60	0.56
0.10	0.67	0.62
0.50	0.70	0.76

Optical density of blank tube containing no thiamine, sulfate, or methionine = 0.13.

III). The total ineffectiveness of cystine and glutathione parallels the fact that these two compounds are inadequate as sulfur sources for yeast, even in the absence of inhibitors. Since sulfate and methionine are equivalent as sulfur sources (Table IV), it would appear that thiamine is syn-

thesized equally as fast from either sulfur source (Table V), perhaps through methionine as an intermediate when sulfate is employed.

Oxidation of Essential Cellular Components

The possibility exists that the mode of action of selenate rests at least in part on its oxidative capacity. This suggestion was proposed previously (1) when it was found that methionine was capable of reacting *in vitro* with H_2SeO_4 or SeO_2, the products obtained depending upon the experimental conditions. Three such reaction products (12) which contained selenium were tested as sources of sulfur for yeast. It was found that, although they produced excellent growth, they varied in their ability to reverse selenate inhibition (Table VI). In contrast to L-methionine,

TABLE VI

Availability of Methionine Derivatives As Sources of Sulfur for Yeast and Their Ability to Reverse Selenate Toxicity

Sulfur source (2 mg.)	Optical density	
	Without selenate	With selenate (1 mg.)
DL-Methionine	1.1	0.73
D-Methionine	0.75	0.00
DL-Methionine sulfoxide	1.1	0.54
" sulfone	0.71	0.00
Compound I-80	1.1	0.45
" I-123	1.0	0.70
" I-119	0.87	0.00

Incubation time, 40 hours.

Compound I-119, as well as methionine sulfone and D-methionine, was completely without effect upon selenate. It would appear, therefore, that the primary action of selenate does not involve the oxidation of methionine to any of the above products. The several B vitamins are similarly unaffected (with the exception of thiamine) because it was found that with methionine as the source of sulfur none of them was able to enhance the reversing effect of methionine in the following maximum concentrations: biotin (0.05 γ), nicotinic acid (10 γ), pantothenic acid (10 γ), riboflavin (10 γ), pyridoxine (10 γ), p-aminobenzoic acid (10 γ), inositol (250 γ), and folic acid (10 γ).

Detoxication of Selenate by Cell

In considering the possible action of the cell upon selenate, a pertinent finding is that of Challenger and North (13) that the mold *Penicillium brevicaule,* when incubated with bread crumbs containing selenite or sele-

nate, produced dimethyl selenide which was isolated and identified as the insoluble mercuric chloride complex.

A procedure similar to that devised by Challenger and North was used for the attempted isolation of dimethyl selenide. Two 12 liter round bottomed flasks, containing 6 liters each of basal medium (1) in which sulfate was replaced by methionine (2 gm. per flask), were connected in parallel to two gas-washing bottles containing 200 ml. of Biginelli's solution[1] (14). To each flask was added 1 gm. of H_2SeO_4, after which the pH was adjusted to 5.2 and the flasks were sterilized by steaming for 45 minutes at 100°. Upon cooling, 500 ml. of a washed cell suspension obtained from a 24 hour culture grown on the basal medium were added to each flask. All stoppers and connections were sealed with paraffin. Air, sterilized by bubbling through concentrated H_2SO_4 and then distilled water, was passed into the flask by means of coarse bubblers. This was continued for 10 days at room temperature. At the end of this time no precipitate was observed in the gas-washing bottles, although growth in the flasks was accompanied by a bright orange-red coloration. Upon breaking the seals, a foul odor was evident. When the experiment was repeated in an atmosphere of nitrogen, the same results were obtained. It seems evident, therefore, that dimethyl selenide is not produced in significant amounts by yeast under these conditions.

Formation of Elementary Selenium

The appearance of an orange-red coloration accompanying growth in the presence of selenium salts has often been recorded in the literature (15-17) and has been observed in all of the present experiments of long duration. The colored substance is intracellular and cannot be extracted (18). It has been assumed to be elementary selenium, and although no actual proof of this has been demonstrated, the fact that it can be decolorized by washing with KCN (18) or by sodium polysulfide supports this assumption.

The reddish coloration can be obtained in the absence of any nutrient substrate by simply adding selenate to a washed yeast suspension in phosphate buffer. The red color develops after 48 hours and increases in intensity upon longer incubation. This is not decolorized by washing. Microscopic examination of yeast cells which have been incubated in the basal medium reveals the presence of large reddish intracellular granules, which gradually increase in number until after 10 days the cells are almost completely opaque.[2]

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[1] The composition of Biginelli's solution is as follows: 10 parts of $HgCl_2$, 20 parts of concentrated HCl, and 80 parts of H_2O.

[2] Photomicrographs of these cells are available for loan upon request from the author's laboratory.

DISCUSSION

The action of methionine in selenate detoxication does not appear to take place through oxidation to sulfate under the specified conditions, although the observation that added sulfate cannot be recovered makes a definite conclusion impossible. In attempting to detect formed sulfate, the assumption is made that such sulfate would be found extracellularly. If this does not occur, it is clear that no sulfate could be detected as $BaSO_4$.

Further evidence that sulfate is not formed from methionine in yeast is obtained from the similar oxygen uptake of cells in the presence and absence of methionine. The minimum quantity of oxygen required, calculated from the following equation,

$$S^- + 2O_2 \rightarrow SO_4^-$$

to oxidize 900 γ of methionine at standard temperature and pressure is 0.35 mg. The actual amount observed, assuming no experimental error, is 0.0016 mg. If any other mechanism is assumed, considerably more oxygen would be required for the oxidation of carbon residues. From these calculations, it seems unlikely that any oxidation has taken place. Finally, an oxidative scheme is evidently not required, since it has been shown that the detoxication can be readily accomplished under anaerobic conditions.

The failure to observe any methylation in the detoxication of selenate suggests that yeast, like certain bacteria (19, 20), but unlike animals, may be unable to demethylate methionine. It has been shown previously (1) that yeasts are incapable of utilizing homocystine plus a methyl donor in place of methionine in reversing selenate inhibition. Although other substrates (e.g., homocysteine) were not tested, it appears that a detoxication mechanism based on methylation is not applicable to yeast.

The ability of cysteine and thiamine to enhance the effect of methionine indicates that one action of selenate may be to block the synthesis of cysteine and thiamine from methionine. This is supported by the equivalence of sulfate and methionine as sulfur sources, and the equal use of either compound as a source of sulfur for thiamine synthesis. However, the fact that thiamine and methionine are not able to overcome completely selenate toxicity when larger amounts of this inhibitor are used indicates that either (a) the synthesis of another essential sulfur compound from methionine is prevented at higher levels of selenate, or (b) this inhibitor has additional effects upon the cell which are manifested at high levels. In view of the equal growth-promoting effects of sulfate and methionine in the absence of inhibitors, the first possibility seems unlikely. The second suggestion appears more probable, especially in view of the observed formation of intracellular deposits, presumably of elementary selenium. The accumulation of this material resulting from its insolubility and non-diffusibility could be expected to interfere with normal

functioning and finally lead to cellular destruction. It can also explain the observation of Potter and Elvehjem (21) that selenium salts become more toxic with time, and finally it can account for the toxic nature of selenium compounds in general. Whereas it is possible that the toxic action of selenate and selenite might be due to oxidation of compounds other than methionine, this could not account for the toxicity of seleno-cystine, diselenodiacetic acid, and other organic selenium compounds (22, 23). The intracellular accumulation of elementary selenium from such compounds thus furnishes one means by which such substances exert their harmful effect. The observation that the intracellular material can be obtained in the absence of nutrient substrate indicates that its formation is independent of the exogenous metabolism and probably involves a cellular component. Whether this component is the enzyme postulated by Rosenfeld and Beath (2) is not certain.

In our opinion, the over-all toxic effect of selenate in yeast is a multiple one in which more than one system is attacked. Two of these undoubtedly involve methionine and thiamine. When sulfate is the sole source of sulfur, the principal point of attack appears to be prevention of methionine synthesis (3). When methionine is present, the toxicity may be due to interference with its utilization, to alteration of methionine residues in enzymes, or to both. Although no mechanism for methionine utilization is offered, it is desirable at this point to recall the specificity of methionine action toward selenate: L-methionine functions both in growth promotion and detoxication, whereas D-methionine and methionine sulfone serve only as growth promoters. Whether this may be due to specific selenate-decomposing enzymes (2) is uncertain, but it appears more likely on the basis of the present study that the activity of the L isomer may be due simply to its preferential participation in enzyme systems in general. It is also of interest to recall the isolation by Horn and Jones (24) of a selenium compound, $C_7H_{14}N_2O_4Se$, which is envisaged by them as the selenium analogue of cystathionine, occurring in mixed crystals with the sulfur analogue in toxic plants. Cystathionine is considered to be an intermediate in the formation of cysteine from methionine in animals (25) and of methionine from cysteine in *Neurospora* (26). If the formation of this selenium compound is of general occurrence, the interference with methionine utilization in yeast and cysteine utilization in *Escherichia coli* (27) can be readily understood.

SUMMARY

1. The rôle of methionine in reversing selenate toxicity has been investigated. Evidence is presented which makes it improbable that the detoxication involves the oxidation of methionine to sulfate, or the methylation of selenate by methionine.

2. Equal growth is obtained with methionine and with sulfate, indicating that all essential cellular sulfur compounds can be synthesized equally well with sulfur from either source.

3. Thiamine and cysteine are the only organic sulfur compounds tested which are capable of enhancing the reversal of selenate by methionine.

4. D-Methionine and DL-methionine sulfone have been found available to yeast as sulfur sources, but are incapable of effecting selenate reversal.

5. The toxicity of selenium compounds is probably a multiple one in which several systems are attacked. Two of these involve methionine and thiamine. A third injurious effect is the intracellular accumulation of an insoluble, non-diffusible substance which is probably elementary selenium.

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SYNTHESIS OF ACETYLCHOLINE IN CRUSTACEAN NERVE AND NERVE EXTRACT*

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The acetylcholine (Ach) synthesizing system, choline acetylase (ChAc), has been rather intensively studied in the vertebrate nervous system (1, 2). Nachmansohn considers the efficiency of the synthesizing system adequate to account for the restoration of Ach that is split during the passage of impulses in the vertebrate nerve. This, he feels, supports his concept that Ach breakdown is a primary phenomenon in the impulse. Gerard (3) has vigorously questioned this conclusion. In view of the small amount of information about invertebrate Ach systems, it seems worth while to report the demonstration of the ChAc system in the nervous tissue of crayfish and crab, thus corroborating the statement of Nachmansohn (4) that this system is present in invertebrate as well as in vertebrate nerve tissue.

Aerobic synthesis of Ach in intact isolated nerve cords of *Cambarus virilis* is recorded in Table I. Nerve cords from two or three crayfish were pooled in each case. The tissue was put in Warburg vessels and incubated in van Harreveld's solution at 16° for 2 hours. Determinations of total Ach levels were made by standard methods, the heart of *Venus mercenaria* being used as a test object (5, 6). The tissue to be tested was thoroughly ground with sand, and Ach was freed with dilute HCl. During the incubation period, somewhat more than a 2-fold increase in total Ach clearly took place in the normal cases (Table I). An attempt was made to determine whether the profound effect of 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane (DDT) upon the activity of the nervous system might be associated with a measurable influence upon the ChAc system. No consistent effect of DDT emulsion or DDT in ethanol was indicated by the results.

The experiments on anaerobic synthesis of Ach in acetone powder preparations of crab nerve are summarized in Table II. For extraction, the central nerve "ring" and leg nerves of several crabs (*Cancer irroratus*) were dissected into ice-cold acetone and ground with sand. The material appears as a fine white powder after collection in a Büchner funnel. To extract the synthesizing enzyme, the powder was ground at +2° in a

TABLE I

Acetylcholine Synthesis in Excised Nerve Cords of Cambarus virilis

Each value represents a separate experiment. The second column shows the amounts of Ach present after a 2 hour period. Total Ach in micrograms per gm. of wet tissue.

Initial Ach	Final Ach
20	65
20	45
25	40
20	45
16	40
30	55
30	55
30	47
20	53
Average ± s.d...22 ± 4	49 ± 7

TABLE II

Acetylcholine Synthesis in Acetone Extract of Nerve Tissue

All syntheses were made in presence of substances indicated in the text plus those shown in the table. Each value represents an individual experiment run for 30 minutes. The Ach synthesis is calculated on an hourly basis.

Activator	Rat juice	Crab juice	Adenosine triphosphate	Ach in acetone powder
		Rat brain		
				γ *per gm.*
0	0	0	0	10
+	+	+	+	900
+	0	+	+	360
0	0	+	+	340
0	0	+	+	200
+	0	0	+	400
+	+	0	+	420
+	+	0	+	420
0	+	0	+	430
		Crab nerve tissue		
+	+	+	+	100
+	+	+	+	90
+	+	+	+	85
+	0	+	+	100
0	0	+	+	200
0	0	0	+	45
+	+	+	0	80
Initial (all additions)... 0–1				

medium of 0.22 M NaCl and 0.015 M KCl with 0.5 M NaHCO$_3$ added to give about pH 7 to 7.2. A sufficient volume of this solution was used to give a final concentration of about 50 mg. of powder per ml. of fluid. For incubation, substances were added in the following final concentrations: 0.04 M NaF, 0.012 M eserine, 0.017 M choline, 0.01 M sodium acetate, 0.02 M L-cysteine, 0.01 M sodium citrate. As indicated, activator (a liver extract kindly supplied by Dr. Fritz Lipmann), adenosine triphosphate (about 5 × 10^{-3} M), and boiled muscle or nerve juice were added to the medium, according to the recommendations of Nachmansohn (7) and Feldberg (8). The boiled juice was prepared by boiling the tissue with an equal volume of water for about 5 minutes. The mass was then ground up and centrifuged. 0.1 ml. of the activator or boiled juice was used as indicated. The adenosine triphosphate was prepared according to the method of Needham (9) with modifications suggested by Dr. Lipmann. The total final volume was 1.5 ml. This small volume was permissible because the *Venus* heart is sensitive to very small amounts of Ach. According to the results of initial control experiments, the added substances had no observable effect on the *Venus* heart in the final dilutions. In studies of the enzyme system, Warburg manometers were at first used. Thunberg tubes were subsequently found more convenient. The atmosphere was 5 per cent CO$_2$ and 95 per cent N$_2$; the temperature 28°. Synthesis experiments were always run with rat brain acetone powder as a control at the same temperature. Although the data of vertebrate and crustacean material are not directly comparable, one would expect that further comparisons would bear out the result that the extract of crustacean nerve tissue synthesizes Ach at a slower rate than does vertebrate tissue. The rat brain synthesis at 28° compares favorably with Nachmansohn's data obtained at 37°.

SUMMARY

The acetylcholine (Ach) synthesizing system has been examined in crustacean nerve tissue, the heart of the clam *Venus mercenaria* being used as test object. In aerobic incubation of whole isolated nerve cords of the crayfish *Cambarus virilis*, the total Ach content rose from 22 ± 4 to 49 ± 7 γ per gm. of wet tissue during a 2 hour period. In anaerobic incubation of acetone powder of nervous tissue of the crab *Cancer irroratus*, between 45 and 200 γ of Ach per hour per gm. of powder were obtained when the saline-extracted powder was incubated in the presence of different activators and adenosine triphosphate.

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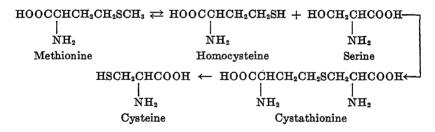
CONVERSION OF CYSTATHIONINE LABELED WITH S³⁵ TO CYSTINE IN VIVO*

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Experiments with methionine labeled with S^{34} and C^{13}, the latter in the β and γ positions, have demonstrated (1) that the carbon chain of methionine is not utilized in the conversion *in vivo* of methionine to cystine. This limits the feasible mechanisms for this transformation to those involving only the sulfur of methionine (2–4). Of such mechanisms, a modification of that of Brand, Block, Kassell, and Cahill (2) has received considerable experimental support in this and other laboratories (5–10). On the basis of these studies, the metabolic pathway may be conceived to be the following.

$$HOOCCHCH_2CH_2SCH_3 \rightleftarrows HOOCCHCH_2CH_2SH + HOCH_2CHCOOH$$
$$\underset{\substack{| \\ NH_2 \\ \text{Methionine}}}{} \quad \underset{\substack{| \\ NH_2 \\ \text{Homocysteine}}}{} \quad \underset{\substack{| \\ NH_2 \\ \text{Serine}}}{}$$

$$HSCH_2CHCOOH \leftarrow HOOCCHCH_2CH_2SCH_2CHCOOH$$
$$\underset{\substack{| \\ NH_2 \\ \text{Cysteine}}}{} \quad \underset{\substack{| \quad\quad\quad\quad | \\ NH_2 \quad\quad\quad NH_2 \\ \text{Cystathionine}}}{}$$

The present communication offers additional evidence that cystathionine is a metabolic precursor of cystine. For these studies, cystathionine has been synthesized with radioactive sulfur (S^{35}). The synthesis was accomplished according to the series of reactions shown in the accompanying diagram.[1]

For the preparation of benzyl β-chloroethyl sulfide* (I) from barium sulfate*, the procedure described recently by du Vigneaud and coworkers (11) was followed. The benzyl β-chloroethyl sulfide* was then diluted with non-radioactive benzyl β-chloroethyl sulfide and carried through the remaining steps. The preparation of S*-benzyl-DL-homocysteine (II)

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[1] An asterisk (*) is used to indicate the presence of radiosulfur in a compound.

from benzyl β-chloroethyl sulfide* and ethyl sodiophthalimidomalonate, was effected by a modification (12) of the method of Tarver and Schmidt (13). The S^*-benzyl-DL-homocysteine was resolved by conversion to the acetyl derivative (III) and incubation with aniline in the presence of cysteine-activated papain, according to the method of Reed, Kidwai, and du Vigneaud (14). The N-acetyl-S^*-benzyl-L-homocysteine anilide (IV) was reduced with sodium in liquid ammonia and, after removal of the ammonia, the residue was condensed with N-benzoyl-β-chloro-L-alanine methyl ester (V) in absolute ethanol. Acid hydrolysis of this mixture produced L-cystathionine* (VI) in a yield of 70 per cent of the theoretical amount on the basis of the N-acetyl-S^*-benzyl-L-homocysteine anilide. The condensation reaction is similar to that used by Melchior and Tarver (15) for the preparation of S^*-benzyl-DL-cysteine.

$$Na-C-(COOC_2H_5)_2$$
$$|$$
$$C_6H_5CH_2S^*CH_2CH_2Cl \xrightarrow{\quad N(CO)_2C_6H_4 \quad}$$
$$(I)$$

$$C_6H_5CH_2S^*CH_2CH_2C(COOC_2H_5)_2 \xrightarrow[\text{(2) HCl}]{\text{(1) NaOH}}$$
$$|$$
$$N(CO)_2C_6H_4$$

$$C_6H_5CH_2S^*CH_2CH_2CHCOOH \xrightarrow[\text{(CH}_3\text{CO)}_2\text{O}]{\text{NaOH}}$$
$$|$$
$$NH_2$$
$$(II)$$

$$C_6H_5CH_2S^*CH_2CH_2CHCOOH \xrightarrow[\text{Papain}]{\text{Aniline}}$$
$$|$$
$$NHCOCH_3$$
$$(III)$$

$$C_6H_5CH_2S^*CH_2CH_2CHCONHC_6H_5 \xrightarrow[\text{(2) ClCH}_2\text{CHCOOCH}_3]{\text{(1) Na liquid NH}_3}$$
$$|$$
$$NHCOCH_3$$
$$(IV) \qquad\qquad NHCOC_6H_5$$
$$(V)$$

$$\text{(3) HCl}$$

$$HOOCCHCH_2S^*CH_2CH_2CHCOOH$$
$$| \qquad\qquad\qquad |$$
$$NH_2 \qquad\qquad\qquad NH_2$$
$$(VI)$$

This cystathionine labeled with S^{35} was fed to two rats at a level of 20 mg. per 100 gm. of diet for a period of 23 days. The cystine was iso-

lated from the hair grown during this period and was found to be radioactive. The cystine was then converted into 4-thiazolidinecarboxylic acid and the latter compound was recrystallized twice from water-ethanol. The radioactivities of the cystine and the 4-thiazolidinecarboxylic acid were found to be the same, within experimental error.

<div style="text-align:center">EXPERIMENTAL</div>

Synthesis of L-Cystathionine Labeled with S^{35}

Benzyl β-Chloroethyl Sulfide*—A solution consisting of sodium sulfide* and sodium sulfate* served as the starting material.[2] A solution of non-radioactive sodium sulfide was added to give a total of 16 mg. of sulfur*. The entire sample was oxidized to sodium sulfate* for use in the present synthesis. The sodium sulfate* was converted to barium sulfate* (126 mg.), which was isolated according to the directions of Kolthoff and Sandell (16). The barium sulfate* was converted to benzyl β-chloroethyl sulfide* according to the procedure of du Vigneaud and coworkers (11). The benzyl β-chloroethyl sulfide* was obtained as a yellow oil and was used without further purification.

S*-Benzyl-DL-homocysteine—The benzyl β-chloroethyl sulfide* described in the preceding section was diluted with 5.43 gm. (0.0291 M) of non-radioactive benzyl β-chloroethyl sulfide. *S*-Benzyl-DL-homocysteine* was then prepared by treatment of the thio ether with 10.0 gm. (0.0306 M) of ethyl sodiophthalimidomalonate according to the method of Kilmer and du Vigneaud (12). The yield of *S*-benzyl-DL-homocysteine* was 4.22 gm. (64 per cent of the theoretical amount). The radioactivity of the product was 7.7 × 10^5 c.p.m. per mg. of sulfur.

N-Acetyl-S*-benzyl-DL-homocysteine—4.8 gm. (0.021 M) of *S*-benzyl-DL-*homocysteine were dissolved in a mixture of 10.6 ml. of 2 N sodium hydroxide and 6.3 ml. of water. The solution was cooled in an ice bath and stirred continuously during the addition of 50.8 ml. of 2 N sodium hydroxide and 5.08 ml. of acetic anhydride over a period of 15 minutes. The mixture was stirred for an additional 30 minutes at room temperature and then was acidified with 22.0 ml. of 6 N sulfuric acid. The mixture was allowed to stand in the refrigerator overnight. It was filtered and the precipitate was washed with cold 0.2 N hydrochloric acid and then with water. The crude product (5.36 gm.) was dissolved in 65 ml. of 15 per cent ethanol, decolorized with norit, cooled, and filtered. 5.07 gm. of crystalline product (89 per cent of the theoretical amount) were obtained.

The material melted at 113.5–114.5°,[3] a melting point identical with
that observed for non-radioactive N-acetyl-S-benzyl-DL-homocysteine (14).

N-Acetyl-S*-benzyl-L-homocysteine Anilide—To 5.07 gm. (0.019 M) of
N-acetyl-*S**-benzyl-DL-homocysteine in a 250 ml. flask were added 12.9
ml. of 1.08 N sodium hydroxide and 3.44 ml. (0.038 M) of aniline. The
material was brought into solution by the addition of 100 ml. of 0.2 M
citrate buffer (pH 5.0) followed by warming the mixture. The solution
was then cooled to room temperature and 0.286 gm. of L-cysteine hydro-
chloride dissolved in 10 ml. of buffer was added. A solution obtained by
extracting 1.44 gm. of papain (dried papaya latex)[4] with 14 ml. of water
was introduced, followed by additional buffer. A total of 145 ml. of buffer
was used. The flask was stoppered, shaken thoroughly, and placed in a
room maintained at 40° for 3 days. The needle-like crystals of *N*-acetyl-
S*-benzyl-L-homocysteine anilide were collected and washed with water.
The compound was dissolved in hot acetone, the solution was filtered,
and the solvent was removed by evaporation. 2.97 gm. of white solid were
obtained. The material melted at 152.5°, a melting point identical with
that observed for non-radioactive *N*-acetyl-*S*-benzyl-L-homocysteine ani-
lide (14). It was used directly in the synthesis of L-cystathionine*.

N-Benzoyl-β-chloro-L-alanine Methyl Ester—This product was prepared
essentially according to the method used by Karrer and coworkers (17) to
obtain the D isomer. 7.0 gm. (0.04 M) of the hydrochloride of β-chloro-
L-alanine methyl ester, obtained by the procedure of Fischer and Raske
(18), were added to a cold solution of 14.6 gm. of sodium bicarbonate in
58 ml. of water. The solution was cooled and stirred during the addition
of 9.2 gm. of benzoyl chloride over a period of 1 hour. The mixture was
kept alkaline during this operation. It was filtered and the precipitate
was washed with cold water and dried *in vacuo* over phosphorus pentoxide.
The product was recrystallized from *n*-heptane to give 8.32 gm. (86
per cent of the theoretical amount) of felt-like white needles; m.p. 111.5–
113°, $[\alpha]_D^{22} = -19.4°$ (1 per cent in absolute ethanol). Karrer and co-
workers (17) obtained a melting point of 114° and a rotation of $[\alpha]_D^{18} = +16.2°$ (approximately 0.88 per cent solution in absolute alcohol) for the
D isomer.

$C_{11}H_{12}O_3NCl$ (241.7). Calculated, N 5.80, Cl 14.7; found, N 5.82, Cl 14.5

L-*Cystathionine**—2.07 gm. (0.00605 M) of *N*-acetyl-*S**-benzyl-L-homo-
cysteine anilide were added to approximately 30 ml. of liquid ammonia
(distilled from sodium) in a tube fitted with a bubbler for the introduction

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[3] All melting points are corrected micro melting points.

[4] The papain was generously supplied by the Wallerstein Company, Inc., New
York.

of dry nitrogen gas and surrounded by a dry ice-cellosolve bath. While a slow stream of nitrogen gas was passed through the mixture, sodium wire was added in small portions until a blue color was produced which persisted for several minutes. A total of 291 mg. (0.0126 M) of sodium was used. The cooling bath was removed and the passage of nitrogen gas through the mixture was continued until all of the ammonia had been removed. 2.92 gm. (0.0121 M) of N-benzoyl-β-chloro-L-alanine methyl ester and 10 ml. of absolute ethanol were introduced and the mixture was heated at 65–75° for 30 minutes. A nitroprusside test on a small sample of the reaction mixture was negative. The mixture was heated under a reflux with 125 ml. of 6 N hydrochloric acid for 12 hours, cooled overnight, and filtered. The precipitate was washed with cold water. The filtrate and washings were combined and concentrated to dryness *in vacuo*. The residue was dissolved in approximately 20 ml. of 1 N hydrochloric acid and filtered through a layer of norit. The pH of the filtrate was adjusted to 6.0 to 6.5 with ammonia and 20 ml. of ethanol were added. The mixture was cooled in the refrigerator overnight and filtered, and the precipitate was washed with water, alcohol, and ether. It was redissolved in approximately 12 ml. of 1 N hydrochloric acid and filtered through norit. The pH of the filtrate was adjusted to 6.5. 10 ml. of ethanol were added and the mixture was cooled for several hours and filtered. The precipitate was washed as already described. The yield was 1.12 gm. (83 per cent of the theoretical amount), $[\alpha]_D^{22} = +22.0°$ (1 per cent solution in 1 N hydrochloric acid). The product gave a positive test with sodium nitroprusside after being treated first with a 5 per cent solution of sodium cyanide. After a second recrystallization from 1 N hydrochloric acid, the test for disulfide was negative. The yield was 0.94 gm. (70 per cent of the theoretical amount), $[\alpha]_D^{22} = +23.4°$ (1 per cent in 1 N hydrochloric acid). Du Vigneaud and coworkers (5) obtained a rotation of $[\alpha]_D^{22} = +23.7°$ (1 per cent in 1 N hydrochloric acid) for non-radioactive L-cystathionine.

$C_7H_{14}O_4N_2S$ (222.3). Calculated, S 14.4; found, S 14.4

The radioactivity of the cystathionine on the 1st day of administration to the rats was 4.2×10^5 c.p.m. per mg. of sulfur.

Conversion in Vivo of Cystathionine to Cystine

Feeding Experiment—Two male rats (Rats 484 and 487), weighing 47 and 48 gm. respectively, were permitted to feed *ad libitum* for 3 days on a basal diet of the following percentage composition: casein 18, hydrogenated vegetable oil (Covo) 19, corn oil 1, Osborne and Mendel salt mixture (19)

4, choline chloride 0.2, sucrose 57.8. The vitamins were supplied in the diet in the quantities described previously (20). The oil-soluble vitamins were dissolved in the corn oil and the water-soluble vitamins were admixed with part of the sucrose. At the end of the 3rd day the animals were anesthetized with ether and the hair removed with a depilatory (Neet).

On the 4th day the basal diet was supplemented with the radioactive cystathionine at a level of 20 mg. per 100 gm. of diet. The animals fed freely on this diet until the 11th day, when the amount of food was restricted to 10 gm. per day. The animals were maintained on the radioactive diet for a total of 23 days. The total amount of L-cystathionine ingested during this time amounted to 45 mg. for Rat 484 and 43 mg. for Rat 487, and the total food intakes were 225 gm. and 214 gm., respectively. The changes in body weight were 78 gm. (Rat 484) and 73 gm. (Rat 487). The animals were then sacrificed and the hair was removed from the carcasses by clipping as closely as possible to the skin.

Isolation of Cystine from Hair—The hair removed from Rats 484 and 487 amounted to 1.2 and 1.3 gm., respectively. It was washed consecutively with water, hot 95 per cent ethanol, and ether, and air-dried on a Büchner funnel. 250 mg. of each rat's hair were hydrolyzed by heating under a reflux for 22 hours with 10 ml. of a 1:1 hydrochloric acid-formic acid mixture. The hydrolysates were concentrated to dryness and the residues were dissolved in a few ml. of water and neutralized to pH 4.5 with 10 per cent potassium hydroxide. The solutions were transferred to centrifuge tubes and the cystine was precipitated as the cuprous mercaptide with cuprous chloride. The procedure for the isolation of the cuprous mercaptide was essentially that described by Rossouw and Wilken-Jorden (21). The cuprous mercaptides were washed twice in the centrifuge tube with 8 ml. portions of ethanol and then suspended in 10 ml. of 0.1 N hydrochloric acid. The suspensions were warmed to boiling and hydrogen sulfide was passed through them for about 30 minutes. The mixtures were then boiled to remove excess hydrogen sulfide, and treated while hot with about 30 mg. of norit. The mixtures were cooled and centrifuged and the supernatant liquids were filtered. The filtrates were concentrated to dryness and the residues were dissolved in a few ml. of water. The cysteine in these solutions was oxidized by "titration" with a 0.06 N alcoholic iodine solution. The cystine solutions were adjusted to pH 5 with 10 per cent potassium hydroxide and diluted with an equal volume of 95 per cent ethanol. Crystals of cystine appeared and the suspensions were placed in the ice box for a few days.

The cystine from each batch was collected, washed with small amounts of water and ethanol, and then dried *in vacuo*. The yields were 24 and 25 mg. of cystine from the hair of Rats 484 and 487, respectively. The sulfur

content of the cystines was determined gravimetrically by micro-Carius combustion and precipitation as benzidine sulfate (22).

$C_6H_{12}O_4N_2S_2$.   Calculated, S 26.7; found, S 25.4 (Rat 484)
240.3   "   " 26.7;   "   " 24.8 ("   487)

The analyses indicated that the cystines were 95 and 93 per cent pure, respectively. The specific radioactivities of the isolated cystines are given in Table I.

TABLE I

Specific Radioactivity Measurements

The specific radioactivity of the cystathionine determined on the same day was 1.95×10^5 c.p.m. per mg. of sulfur.

Rat No.	Counts per min. per mg. sulfur		
	Cystine	4-Thiazolidine-carboxylic acid	4-Thiazolidine-carboxylic acid
		1st recrystallization	2nd recrystallization
484	2460	2430	2450
487	2580	2640	2580

In preliminary experiments in which non-radioactive cystine was isolated from normal rat hair in amounts similar to those obtained in the isotope experiments, the cystine possessed about the same degree of purity as did the radioactive cystine. Repeated solution of the non-radioactive cystine in a small volume of 0.1 N hydrochloric acid and reprecipitation with either 0.1 N sodium hydroxide or 0.1 N ammonium hydroxide did not change the analytical values appreciably, although there was a substantial loss of material. Therefore, the radioactive cystine was converted to cysteine by reduction with tin and hydrochloric acid and the cysteine was transformed to 4-thiazolidinecarboxylic acid. This conversion of cystine to 4-thiazolidinecarboxylic acid offers an excellent opportunity for removal of radioactive impurities, if any are present.

Conversion of Cystine to 4-Thiazolidinecarboxylic Acid—The preparation of 4-thiazolidinecarboxylic acid from cysteine and formaldehyde was carried out according to the method described by Ratner and Clarke (23). The cystine was first reduced to cysteine by the following procedure. 20 mg. of cystine were dissolved in 0.5 ml. of 6 N hydrochloric acid in a short test-tube, and to the solution were added 400 mg. of mossy tin and 0.01 ml. of a 1 per cent solution of platinum chloride. The mixture was heated for 2 hours on a steam bath, while the mouth of the test-tube was covered with a 10 ml. pear-shaped flask filled with water to act as a reflux

condenser. Then the excess tin was removed and the solution was concentrated almost to dryness on the steam bath under a stream of nitrogen. The tube and its contents were placed overnight in an evacuated desiccator with phosphoric anhydride and potassium hydroxide. The residue was dissolved in 5 ml. of water. The solution was warmed and hydrogen sulfide was passed through it for 1 hour. The stannous sulfide was separated by centrifugation and the supernatant was filtered through a layer of Hyflo Super-Cel. The filtrate was concentrated to dryness in portions in a 2 ml. centrifuge cone, under a stream of nitrogen. To the residue were added 0.5 ml. of water, 0.05 ml. of 35 per cent formaldehyde (Baker's c.p. grade), and 0.03 ml. of pyridine. Crystals appeared in about 2 hours. About 0.1 ml. of 95 per cent ethanol was added and the mixture was cooled overnight. The crystals were separated by centrifugation, washed twice with 0.2 ml. portions of 95 per cent ethanol, and dried *in vacuo* at room temperature over phosphoric anhydride. The yield was 13 mg. The material was recrystallized as long slender needles by dissolving it in 0.2 ml. of hot water and cooling. An equal volume of 95 per cent ethanol was added and the mixture was cooled for a short time in ice. The crystals were separated, washed, and dried as before. The yield was 12 mg. The sulfur content of the 4-thiazolidinecarboxylic acids was determined by conversion to sulfate by micro-Carius combustion and by precipitation as the benzidine sulfate.

$C_4H_7O_2NS$. Calculated, S 24.1; found, S 24.4 (Rat 484)
133.2   "   " 24.1; "   " 24.2 ("   487)

The precipitates of benzidine sulfate used for the sulfur analyses were redissolved in a slight excess of alkali, reprecipitated, and filtered for the determination of radioactivity. The specific activities of the recrystallized thiazolidines are given in Table I. The thiazolidines were recrystallized a second time as described above and the radioactivities of the twice recrystallized samples are given in Table I.

Radioactivity Determinations—Samples of the *S*-benzyl-DL-homocysteine, the L-cystathionine, the isolated cystines, and the 4-thiazolidinecarboxylic acid preparations were converted to sulfate by the micro-Carius method. In the case of the *S*-benzyl-DL-homocysteine and the L-cystathionine, the resulting digests were diluted and aliquots were taken for measurements of the radioactivity. Carrier sulfate was added to these aliquots to provide sufficient precipitable material. In the other cases, the sulfur of the compounds provided all the precipitable sulfate. The sulfates were precipitated with benzidine hydrochloride and the precipitates were collected on weighed filter paper disks by the method described by Henriques and coworkers (24). The radioactivity of the precipitates was determined by

means of a Geiger-Müller counter tube with a thin mica window of 1.85 mg. per sq. cm. thickness. The scaling circuit used in conjunction with the counter was an autoscaler,[5] mark II. A total count of 4096 was obtained for each sample, and the activities in counts per minute were corrected for background count and for self-absorption by the use of a self-absorption curve prepared in this laboratory.

DISCUSSION

It may be recalled that in an earlier experiment (1), in which methionine labeled with the stable isotopes S^{34} and C^{13} was administered in the diet as the sole sulfur-containing amino acid during a period of 35 days, the S^{34} content of the isolated cystine was 80 per cent of the S^{34} content of the fed methionine. In the present experiment, a small amount of radiocystathionine was added to a casein diet and thus the amount of cystathionine was small in comparison with the amount of methionine and cystine in the diet. The casein in the diet was assumed to contain 3.5 per cent of methionine and 0.36 per cent of cystine (25). The labeled sulfur fed as cystathionine represented approximately 1.9 per cent of the total sulfur intake of the rats during the course of the experiment. The radioactivities of the isolated cystine and the fed cystathionine indicate that the specific activity of the sulfur of the cystine is 1.3 per cent of that of the sulfur of the cystathionine. The ratio of the specific activity of the sulfur of the cystine to that of the sulfur of the cystathionine is approximately 70 per cent of the ratio of the labeled sulfur of cystathionine to the total sulfur of the diet. In view of the high dilution in the diet of the cystathionine by methionine and cystine, the conversion of cystathionine to cystine in the present experiment appears to be of a high order of magnitude.

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SUMMARY

L-Cystathionine labeled with S^{35} was prepared and fed to rats for a period of 23 days. The cystine was isolated from the hair grown during this period and was found to be radioactive. The cystine was converted to 4-thiazolidinecarboxylic acid and the latter compound was subjected to two recrystallizations. The radioactivities of the isolated cystine and the recrystallized 4-thiazolidinecarboxylic acid were the same, within experimental error. Thus, the formation of cystine from cystathionine in

[5] The autoscaler was manufactured by Tracerlab, Inc., Boston, Massachusetts.

the rat has been corroborated by the use of isotopic sulfur. The results are compared with those obtained in earlier feeding experiments with methionine labeled with S^{34} and with C^{13}.

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RADIOACTIVE CRYSTALLINE RIBONUCLEASE*

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The biological synthesis of a protein might occur either *de novo* from amino acids or by the ordered coupling of amino acids and preformed peptide fragments. The first alternative would require that, following the incorporation of a radioactive amino acid, the specific activity of this amino acid isolated from one part of the protein molecule must be identical with that derived from other parts. The second alternative, however, might lead to differences in these specific activities.

To serve as a suitable working material for an examination of this problem, radioactive ribonuclease has been prepared *in vitro*. The data below demonstrate that this protein may be obtained in a state of sufficient purity to follow phase rule criteria and that the incorporated radioactivity is associated with the enzymatic activity.

EXPERIMENTAL

Bovine pancreas glands were sliced with a 0.5 mm. Stadie slicer at the slaughter-house within 15 to 30 minutes after the death of the animal. Slices weighing 10 gm. were transferred to a 500 cc. Erlenmeyer flask containing salt solution (1) maintained at pH 7.5 with a 40 mM per liter bicarbonate-5 per cent CO_2-95 per cent O_2 buffer system. In most of the experiments about 1 to 3 $\times$ 10⁶ total counts per minute were added as sodium carbonate. The flasks were rocked at 38° for 2 hours. At the end of this period the flasks were chilled in ice, and ice-cold 0.5 N sulfuric acid was added to give approximately pH 1.5. During this addition and for 1 hour after the required pH had been reached, a stream of air was passed through the flask and the carbon dioxide was reabsorbed in barium hydroxide for use in subsequent experiments.

After homogenizing the flask contents, the insoluble débris was centrifuged at 0°. The resulting turbid supernatant was treated as described by Kunitz (2) for the isolation of ribonuclease. Precipitates were collected by high speed, angle centrifugation rather than by filtration because of the small amounts of material involved. The first batch of crude crystalline material was assayed for ribonuclease activity by the method of Kun-

itz (3). On the basis of these analyses and of analyses for total amino acid content by the ninhydrin method (4), the material at this stage was roughly of 60 to 80 per cent purity and amounted to about 5 to 10 mg. In several preliminary experiments, these fractions were precipitated and washed with trichloroacetic acid and alcohol-ether and subjected to acid hydrolysis. The hydrolysates were treated with ninhydrin and the resulting barium carbonate was counted (5, 6). These experiments indicated, as shown in Table I, that the specific activity of the constituent amino acid carboxyl groups was about 0.1 per cent that of the gas phase carbon dioxide and that the rate of incorporation was about twice that of the average slice protein.

In order to obtain sufficient material for the distribution studies referred to above and to determine whether or not the observed radioactivity was

TABLE I

Radioactivity* of Pancreas Slice Proteins and Isolated Crude Crystalline Ribonuclease

	Experiment I	Experiment II	Experiment III
CO_2 in flask	1×10^6	1×10^6	2.1×10^6
Slice proteins	310	720	390
Ribonuclease preparation	870	1870	716

* The radioactivity is expressed as counts per minute per mM of $BaCO_3$, prepared either from the gas phase CO_2 of the incubation flasks or from CO_2 derived from ninhydrin treatment of the hydrolysates of washed, defatted protein.

derived from the ribonuclease molecule and not from adsorbed contaminants, several experiments were carried out in which the carbon dioxide in the flasks was generated entirely from barium carbonate obtained from Oak Ridge. The ribonuclease content of the first crude crystalline precipitate was determined by assaying the enzymatic activity. This small radioactive fraction was then diluted 100-fold by the addition of non-radioactive ribonuclease recrystallized five times.[1]

The resulting material was dissolved in 4 cc. of water and the solution was adjusted to pH 4.8 with 0.1 N NaOH. Solid ammonium sulfate was added to give a marked turbidity. After standing with occasional stirring for ½ hour, the precipitate was centrifuged. The clear supernatant was adjusted to pH 4.3 with 0.25 N H_2SO_4 and saturated ammonium sulfate was slowly added until a thixotropic opalescence appeared. On standing overnight, a heavy crystalline precipitate settled out in the form of thin plates.

[1] The non-isotope ribonuclease used for dilution was obtained from the Armour Laboratories. Chemical, physical, and enzymatic studies on this material indicate a high degree of purity, in good agreement with earlier studies by Kunitz (2), Rothen (7), and Brand (8).

This material was recrystallized three times at pH 4.3. The fine crystalline product was washed with 58 per cent saturated ammonium sulfate, in 0.04 M acetate buffer, pH 4.0, by alternate suspension and centrifugation until the protein content of the washings became constant as measured by light absorption measurements at 2800 A.

The washed crystals were suspended in the same solution which was used for washing. To test the possible presence of adsorbed radioactive
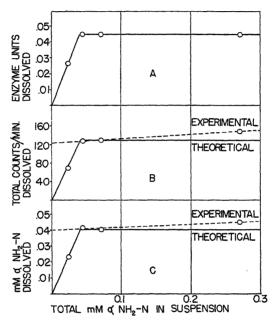
FIG. 1. Comparison of dissolved enzyme activity, radioactivity, and NH$_2$-N in the presence of increasing quantities of solid phase. Radioactive, crystalline ribonuclease equilibrated with 54.9 per cent saturated ammonium sulfate, pH 4.0. Specific radioactivity, (A) control aliquot, 3270 c.p.m. per mM; (B) after extraction with 10 per cent trichloroacetic acid containing 300 mg. per cent each of aspartic and glutamic acids, at 100°, for 5 minutes, 3300 c.p.m. per mM; (C) after extraction with boiling acetone, 3350 c.p.m. per mM.

glutamic and aspartic acids and amino acid containing lipides, aliquots of the crystal suspension were boiled with acetone and with 10 per cent trichloroacetic acid containing non-isotopic glutamic and aspartic acids at a level of 300 mg. per cent. The specific activity of the carbon dioxide liberated by ninhydrin treatment of the hydrolysates of these samples was not significantly different from that derived from control aliquots (see the legend to Fig. 1).

The solubility of the crystalline protein was then determined in the

presence of increasing quantities of the solid phase. In the experiment reported below, volumes of crystal suspension varying from 0.5 to 6.0 cc. were made up to 8.5 cc. with 58 per cent ammonium sulfate at pH 4.0. In order to increase the solubility to a point at which the first tube contained no solid phase, 0.5 cc. of water was added to each tube, resulting in a final ammonium sulfate concentration of 54.9 per cent of saturation.

After rocking for 10 hours at 25°, the tubes were centrifuged at 3000 r.p.m. for 20 minutes. 0.0338 cc. aliquots of the supernatant were diluted to 10 cc. with 0.1 M acetate buffer at pH 5.0 and the enzyme activity was assayed. 7 cc. aliquots were treated with 7 cc. of 20 per cent trichloroacetic acid and, after standing overnight, the precipitated protein was centrifuged, washed twice with trichloroacetic acid, and autoclaved in 6 N HCl for 6 hours. The resulting amino acid solution was neutralized and treated with ninhydrin. The liberated carbon dioxide was collected in $Ba(OH)_2$ and the precipitated $BaCO_3$ washed, weighed, and assayed for radioactivity.

The radioactivity and enzyme activity data from this experiment are presented in typical "phase rule" plots in Fig. 1. The solid lines represent the theoretical curves for a pure substance. In the case of the enzyme activity values (Fig. 1, A) the points fall on the theoretical line. In Fig. 1, B and C, however, it is seen that at high levels of solid phase both the radioactivity and the total amount of protein dissolved fall above the theoretical values in this experiment, although the *specific* radioactivity remains constant. On the basis of the theory of the phase rule test (9), these data indicate an impurity of 2 to 4 per cent. The constancy of the specific radioactivity values at both low and high levels of solid phase suggests that this impurity is denatured ribonuclease produced during the washing and equilibration procedures involved. Subsequent experiments on other preparations have indicated the presence of enzymatically inactive impurities at a level of less than 1 per cent of the total protein.

SUMMARY

1. Radioactive, crystalline ribonuclease has been prepared *in vitro* by incubation of bovine pancreas slices with $C^{14}O_2$.

2. This protein contains incorporated radioactivity at approximately twice the level of the average slice proteins.

3. Following repeated recrystallization of the radioactive enzyme diluted with non-radioactive carrier, a product was obtained which obeyed phase rule criteria of purity within narrow limits. The data suggest that the small amount of impurity present is mainly denatured ribonuclease.

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LABELED SULFUR UPTAKE BY THYROIDS OF RATS
WITH LOW PLASMA THIOCYANATE LEVELS*

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In a previous paper (1) we reported data, based upon the use of radio-sulfur, showing that in the rat the affinity of the whole thyroid for thio-cyanate ion is slight but is significantly greater than in other tissues. Baumann and Metzger (2), using different analytical procedures, reported a similar observation.

The uptake of thiocyanate ion by the thyroid is of especial interest because of the chemical resemblance of the ion to iodide (3), as well as the physiological antagonism to iodide which thiocyanate exhibits. Thio-cyanate is a goitrogen whose effect is reversed by iodide administration (4); it acts on the thyroid to release iodide from the gland, and it interferes with the formation of protein-bound iodine (5, 6). The metabolism of thiocyanate by the thyroid is inhibited by propylthiouracil (1). From such considerations the question has been raised by several investigators (1, 5–9) whether thiocyanate might be accumulated by the thyroid to the degree or in the manner by which iodide is concentrated. The present study was made because the available data were inconclusive on these points.

It has been observed that raising the iodide level of the plasma obscures the normal thyroid-serum iodine gradient, since the capacity of the gland to absorb iodide is limited (10). By analogy the thyroid might be similarly limited in its ability to absorb thiocyanate. This would explain the low concentration gradients in experiments previously reported (1, 2), since the large dosages used produced high plasma levels. It thus seems desirable to investigate the uptake of thiocyanate by the thyroid from very low plasma levels of this ion.

Adult, male rats were prepared by feeding for 33 days a diet of standard laboratory ration, a "low iodine" diet (11), or a propylthiouracil diet. About 0.75 μM of thiocyanate ion labeled with S^{35} was injected into each animal and, after varying intervals of time, the ratios of labeled sulfur between the thyroids and equal weights of serum were determined by radioactivity measurements. The results are shown in Table I. In none

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TABLE I

Uptake of Labeled Thiocyanate Sulfur by Rat Thyroid after Intraperitoneal Injection

Diet	Weight of rat	Weight of thyroid	SCN-S injected	Time interval	Amount in thyroid		Labeled S in serum	Labeled S ratio, thyroid-serum
					Per cent injected $\times 10^3$	S per gm. tissue		
	gm.	*mg.*	γ	*hrs.*		γ	γ *per cent*	
Checkers	79	14	24.6	0.5	17	0.29	84	0.35
	61	17	24.6	0.5	20	0.29	82	0.35
	215	19	23.4	1	16	0.19	29	0.66
	171	11.5	23.4	1	19	0.39	42	0.93
	79	12	24.6	1	11	0.22	46	0.48
	99	14	24.6	1	20	0.28	45	0.62
	77	14.5	24.6	1	16	0.34	64	0.53
	71	10	24.6	1	9	0.34	73	0.47
	181	13	23.4	1	10	0.19	30	0.63
	243	12	23.4	1.5	16	0.31	33	0.94
	140	17	23.4	1.5	19	0.27	38	0.71
	61	12.5	24.6	3	23	0.45	50	0.90
	65	9	24.6	3	10	0.26	61	0.43
	84	11	24.6	3	22	0.41	66	0.62
	101	19	24.6	3	23	0.26	49	0.53
	80	12	24.6	5	18	0.47	50	0.94
	83	12	24.6	5	22	0.46	52	0.88
Average....								0.65 ± 0.05*
Checkers with 0.1% propyl-thiouracil	127	44.5	24.6	0.5	37	0.20	46	0.44
	101	45.5	23.4	1	51	0.26	58	0.45
	118	44.5	23.4	1	47	0.25	28	0.89
	96	36.5	23.4	1	59	0.46		
	104	54	23.4	1.5	70	0.31		
	150	59	23.4	1.5	29	0.12	26	0.45
	120	34	24.6	3	33	0.24	53	0.45
	94	24.5	24.6	3	22	0.21	45	0.47
	133	40.6	24.6	3	27	0.17	40	0.43
Average...								0.51 ± 0.06*
Low iodine	251	20	39	0.5	13	0.26	51	0.51
	159	20.5	39	0.5	20	0.39	70	0.56
	250	19	39	1	16	0.32	50	0.64
	238	18.5	35	1	14	0.26	37	0.70
	203	24	23.4	1.5	14	0.14	32	0.44
	215	17.5	23.4	1.5	11	0.15	32	0.47
	146	15	23.4	1.5	18	0.29	40	0.73
	273	20	23.4	2	24	0.28	31	0.90
	213	18	23.4	2	19	0.26	38	0.68
	171	21	23.4	2	21	0.24	36	0.67
Average....								0.63 ± 0.01*

* ± the standard error.

of the animals was the thyroid-serum ratio of labeled sulfur larger than 1 over periods of 0.5 to 5 hours.

EXPERIMENTAL

Potassium thiocyanate labeled with radioactive sulfur was synthesized shortly before use. A solution of approximately 2 mg. of sulfur in xylene was prepared as previously described (12). The xylene was evaporated to 0.2 ml., 3 ml. of acetone and 50 mg. of potassium cyanide were added, and the solution was heated under a reflux for 1.5 hours. The solution of potassium thiocyanate was filtered and evaporated to dryness *in vacuo*. The residue was dissolved in 5 ml. of water. The thiocyanate content of the solution was determined by the method of Powell (13).

Several weight groups of rats ranging from 60 to 273 gm. were used. They were prepared by feeding for 33 days Purina dog chow checkers, checkers with 0.1 per cent *n*-propylthiouracil, or a low iodine diet which was essentially that of Koenig, Gassner, and Gustavson (11). Thiocyanate solutions were diluted with physiological saline and injected intraperitoneally as soon as possible after preparation to minimize decomposition. The amounts injected (about 0.75 μM) are given in Table I. After varying periods of time, the animals were anesthesized with ether, blood samples were taken from the heart, and the thyroids were removed.

The thyroids and aliquots of the serums were dried and oxidized by the Carius procedure. The sulfate was precipitated with benzidine. The radioactivity was determined by methods previously described (1). Sulfur derived from injected thiocyanate ion was calculated from a comparison of counts of samples and a standard sample.

Two rats which had been maintained on the low iodine diet and two which had been fed Purina checkers were injected with 180,000 c.p.m. of carrier-free I^{131} solution.[1] Blood samples were taken and the thyroids were removed after 20 hours. In each specimen the total radioactivity due to I^{131} was determined. The ratio of thyroid I^{131} to serum I^{131} was 1800 and 1900 for the animals on the checkers diet and was 16,000 and 20,000 for the animals on the low iodine diet.

DISCUSSION

The data of this investigation may be correlated with those of previous work (1, 2). In our previous study we injected 8.33 mg. of thiocyanate sulfur per rat. After 6 hours we observed an average thyroid-plasma ratio of thiocyanate *ion* of 0.58 for a plasma level of 4.4 mg. per cent of labeled sulfur and, after 24 hours, a ratio of 0.56 for a plasma level of 0.43

[1] This experiment was carried out with the collaboration of Dr. L. Van Middlesworth.

mg. per cent.[2] Baumann and Metzger observed thyroid-*blood* ratios of 1.2 to 2 when the blood level of thiocyanate was 16 to 32 mg. per cent of thiocyanate sulfur. In the present experiments, the thyroid-serum ratios of labeled sulfur were 0.35 to 0.95, averaging 0.65 for a serum level of 29 to 84 γ per cent of labeled sulfur. It appears significant that with decreasing serum levels of thiocyanate ion there is no increase in the concentration gradient between the thyroid and the serum.

The labeled sulfur values of the thyroids and serums recorded in Table I represent free thiocyanate ion plus the metabolism products of thiocyanate. By comparison with the experiments of Taurog, Chaikoff, and Feller (14), the percentage of administered dose found in the thyroid was about one-hundredth the uptake of a similar dosage of labeled iodide.

The results of Taurog, Chaikoff, and Feller show that the uptake of labeled iodide by the normal thyroid from an injected dose of 100 γ (0.89 μM) was about one-tenth of that from a tracer dose. Thus, it would have been desirable to perform the present study with tracer amounts of thiocyanate ion. This was regarded as impractical, for it would require synthesis of thiocyanate from carrier-free S^{35} in sufficient amount to permit the characterization of the prepared material by a method independent of radioactivity analysis. Accordingly, a thiocyanate sample was synthesized from labeled sulfur with specific radioactivity high enough to permit radioactivity measurements on 0.5 ml. of serum after injection of 1 μM of thiocyanate ion per animal. After synthesis the amount of thiocyanate ion in the sample was determined by a colorimetric procedure before dilution and injection. This method of synthesis insured to a fair degree of certainty that other species of radioactive compounds were absent from the solution.

It seems likely that a 1 μM dosage of thiocyanate ion would have permitted the thyroid to maintain a high concentration gradient against the plasma if such a gradient could exist (15). This conclusion is in keeping with the observation that the effect of thiocyanate ion on iodide uptake by the thyroid is lost with the disappearance of thiocyanate from the serum (6).

From a consideration of the competition between thiocyanate and iodide in biological systems it seemed possible that a high level of thyroid-fixed iodide in a rat maintained on an adequate diet might block the uptake of thiocyanate ion. To eliminate this explanation for our results experiments were performed with iodine-depleted animals. The efficacy of the low iodine diet was checked by demonstrating that the uptake of carrier-free I^{131} in two depleted animals was 10 times that in two animals on the usual diet. In the other iodine-depleted animals the uptake of thiocyanate sulfur by the deficient thyroids was no larger than by normally nourished glands.

[2] On the basis of *total* labeled sulfur, the thyroid content was 1.2 and 6.5 times that of an equal weight of plasma for the 6 and 24 hour periods respectively.

Similar results were obtained in animals with glands made goitrous by chronic propylthiouracil administration. Taurog, Chaikoff, and Feller (14) found that the usual iodide uptake by the thyroids was increased 20-fold 30 minutes after injection into rats made goitrous with propylthiouracil. Vanderlaan and Vanderlaan (7) found similar results, and showed that no modification in iodide uptake occurred up to 100 γ (0.6 μM) of potassium iodide dosage. The data in Table I show that the goitrous thyroids took up approximately the same amounts of a 0.75 μM dose of thiocyanate ion during the period of 0.5 to 3 hours as did the normal glands.

The data of this study are consistent with the conclusion that the uptake of thiocyanate by the thyroid does not resemble its concentration of iodide in kind or magnitude.

SUMMARY

Approximately 0.75 μM of thiocyanate ion labeled with S^{35} was injected into rats. Determinations of the labeled sulfur in the thyroids and serums were made after intervals of 0.5 to 5 hours. The concentration gradients between the thyroids and serums were of the same magnitude as reported for blood levels up to 1000-fold greater. The percentage uptake of the dose was about one-hundredth the uptake of a similar dosage of iodide. Thyroids made deficient in iodine by a low iodide diet, or by propylthiouracil administration, showed no greater uptake of thiocyanate ions than normal thyroids.

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MICROBIOLOGICAL DETERMINATION OF CYSTINE, CYSTEINE, AND GLUTATHIONE IN PLASMA*

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In previous reports from this laboratory, a method for the assay of D- and L-methionine in biologic fluids with *Leuconostoc mesenteroides* P-60 and *Lactobacillus fermenti* 36 has been described (1, 2). The present communication describes a method for the determination of cysteine plus cystine in plasma.

In a report by other workers, using the medium of Dunn *et al.* (3), the organisms *Lactobacillus brevis* (8257),[1] *Lactobacillus pentoaceticus* (367),[1] *Lactobacillus buchneri*, *Lactobacillus lycopersici* (4005),[1] *Leuconostoc mesenteroides* P-60 (8042),[1] and *Leuconostoc dextranicum* (8359)[1] have been described as requiring cysteine or cystine for adequate growth. These organisms have been methodically evaluated by us in a variety of additional media, including fortified peroxide-treated gelatin or casein hydrolysate (1, 2), the medium of Henderson and Snell (4), and that of Steele *et al.* (5). Only *L. lycopersici* and *L. buchneri* have been found to be satisfactory for quantitative determination of these amino acids under the conditions to be described.

Methods

Assays were carried out in 13 × 100 mm. culture tubes. Each tube received 1 ml. of a basal medium with supplements to a total volume of 2 ml. The standard curve of reference was constructed by the use of a cystine solution containing 5 γ per ml. for *L. lycopersici* or 4 γ per ml.

for *L. buchneri* and by adding 0.0, 0.2, 0.4, 0.6, 0.8, or 1.0 ml. of the standard solution to a series of tubes (Fig. 1). The assay of normal plasma was performed in a similar manner. The protein-free filtrate, prepared as previously described (1), results in a 1:2 dilution of the original plasma. This was further diluted with water to result in a final dilution of plasma of 1:5. 0.5 ml. of this solution was added to each assay tube when *L. lycopersici* was used, or 0.4 ml. when *L. buchneri* was the test organism. Water was then added to all tubes to make a final volume of 2 ml. Samples of abnormal blood which were suspected of containing excessive amounts of cystine were diluted accordingly. All tubes were then covered with a
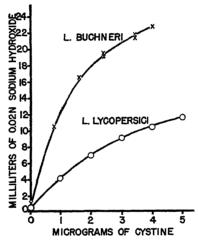
Fig. 1. Standard reference curves for cystine (medium of Steele *et al.*)

towel and were sterilized by autoclaving at *10 pounds for 5 minutes* (*media containing glucose*) *or steaming for 5 minutes* (*media containing arabinose*). After cooling, the tubes were prepared for inoculation as described below.

The stock cultures of the test organisms were maintained on tomato juice agar stabs (Difco) and a 24 hour broth culture for use in the assay was subcultured in an enriched peptone medium. This culture was centrifuged, washed, and resuspended in sterile saline. 1 drop from a sterile pipette was then used to inoculate each assay tube. Incubation at 37° for 72 hours was followed by electrometric titration (of the lactic acid produced) with 0.02 N NaOH. The cystine concentration was estimated by the use of a reference curve in the customary manner. Incubation at temperatures below 37° resulted in decreased acid production; at 32° there was no growth. Good growth was obtained at temperatures as high as 40°.

Results

As stated above, a number of media were evaluated for use in the assay of cystine. Glucose was used as the carbohydrate source for *L. lycopersici* but arabinose was necessary for *L. buchneri* (6). The pentose sugar also enhances acid production by the former organism but results were erratic when compared to those obtained with glucose. The medium of Henderson and Snell (4) supported excellent growth of both organisms if 2.4 gm. of sodium acetate were substituted for the citrate buffer and if the original Salts A and B of Snell and Wright (7) were substituted for the Salts C and KH_2PO_4. Excellent results were also obtained with the medium for *Leuconostoc* recently published by Steele *et al.* (5), the only modification being the use of arabinose instead of glucose when *L. buchneri* was the test organism. When the amino acids of the modified medium of Henderson and Snell were replaced with a supplemented acid hydrolysate of casein or with hydrogen peroxide-treated gelatin, good growth of both organisms resulted.[2]

Both organisms responded identically to cysteine or cystine in equivalent quantities. The basal media contained considerable excesses of methionine (up to 400 γ per tube). The addition of as much as 200 γ of methionine to various concentrations of added plasma filtrates did not produce a deviation of more than ±1.25 per cent in the predicted cystine values. This is within the experimental error of the procedure.

Rabinowitz and Snell (8) and Riesen, Spengler, Robblee, Hankes, and Elvehjem (9) reported that the autoclaving of media containing cystine-cysteine at 15 pounds pressure for 15 minutes resulted in destruction of these amino acids. This has been confirmed in this laboratory.

To determine whether autoclaving at 10 pounds pressure for 5 minutes resulted in any destruction of cystine-cysteine, the following procedures were carried out. A standard reference curve was obtained by the method described above. A second curve was obtained by adding equal amounts of cystine, which had been passed through a Seitz filter, to previously sterilized cystine-free medium. The growth curves were identical.

Because of the presence of glutathione in biological fluids including blood, it was of interest to investigate the response of the test organisms to this cysteine-containing tripeptide.

When glutathione was added to cystine-free medium, and heat steriliza-

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[2] In the preparation of the casein and gelatin hydrolysate media, the following, in amounts per 100 ml. of basal medium, may be used to replace the amino acid mixture of the modified medium of Henderson and Snell: (1) 20 ml. of 5 per cent casein or 20 ml. of 5 per cent peroxide-treated gelatin hydrolysates; (2) 10 mg. of L-tryptophan; (3) 40 mg. of DL-methionine; (4) 10 mg. of L-tyrosine; (5) 40 mg. of L-glutamic acid.

tion was performed as noted above, growth equivalent to the cysteine content of the glutathione occurred.

Repetition of the procedure with glutathione which had been passed through a Seitz filter, resulted in growth which at all times was equivalent to less than 20 per cent of the cysteine content of the added glutathione. These findings suggest that (a) autoclaving under the conditions noted results in hydrolysis of glutathione and (b) the organisms are able only

TABLE I

Concentration of Cysteine Plus Cystine in Plasma of Various Patients
Values in mg. per 100 ml.

Patient	Diagnosis	Test organisms	
		L. lycopersici	*L. buchneri*
ROM	Acute hepatitis, severe	4.6	4.9
SHA	Diabetes mellitus	2.7	2.7
ARM, Aug. 2	Normal	1.8	1.88
" " 3	Receiving methionine	1.6	1.6
" " 9	Post methionine	1.85	1.85
BEL, " 2	Cirrhosis	2.3	2.35
" " 3	Receiving methionine	2.1	2.25
" " 9	Post methionine	3.0	3.2
TUC, " 2	Cirrhosis	1.95	2.0
" " 3	Receiving methionine	1.95	1.95
" " 9	Post methionine	2.55	2.5
GLE	Normal	1.55	1.55
"	Receiving methionine	1.5	1.4
LOP, Aug. 2	Normal	1.9	1.9
" " 3	Receiving methionine	1.75	1.8
BRA	Cholangitis, receiving methionine	2.5	2.4
HOO	Cirrhosis	2.3	2.5
LAR	"	1.85	2.0
WHI	Hepatitis with probable cirrhosis	1.8	1.95
LAN	Acute hepatitis	2.2	2.25

feebly to utilize previously unhydrolyzed glutathione. Consequently the combination of the two procedures provides a method for determination of free cystine-cysteine and of glutathione.

The addition of 10 γ each of homocystine, cystathionine, and homo-lanthionine to cystine- and cysteine-free media, followed by heat steriliza-tion, resulted in no growth.

Cystine-cysteine-glutathione were determined on specimens of blood obtained from different individuals with both test organisms and the various media already described. The values obtained with each organism

on a given blood sample did not differ significantly (Table I). Recovery experiments on human blood plasma were also carried out. Mean recovery values for eighteen separate determinations with *L. lycopersici* were 101 ± 1.4 per cent[3] (extreme values 89 and 109 per cent). The corresponding figures for fourteen separate determinations with *L. buchneri* were 97 ± 0.97 per cent (extreme values 90 and 103 per cent).

SUMMARY

A microbiological method is described for the determination of free cysteine plus cystine and of glutathione-cysteine in human plasma by use of the organisms *L. lycopersici* and *L. buchneri*. Reproducible recoveries were obtained under the various experimental conditions noted. The concentration of total cysteine plus cystine in normal fasting human plasma ranged from 1.55 to 2.0 mg. per 100 ml.

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[3] Standard error of the mean $= \sqrt{(\Sigma d)^2/(n(n-1))}$.

PREVIOUS NUTRITIONAL STATE AND GLUCOSE CONVERSION TO FATTY ACIDS IN LIVER SLICES*

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That carbohydrate utilization depends upon an animal's previous nutritional state has long been recognized and is supported by an overwhelming amount of evidence (1, 2). Thus glucose utilization is depressed in the fasted animal and in the animal fed a diet containing little or no carbohydrate. The feeding of glucose immediately following a period of fasting or chronic undernutrition results in hyperglycemia and glycosuria. This phenomenon, which was first recognized by Claude Bernard, was named "hunger diabetes" by Hofmeister.

Depressed glucose oxidation, depressed glycogenesis, and increased gluconeogenesis have been regarded as the metabolic defects responsible for the impaired carbohydrate utilization observed under these hunger conditions (1, 2). Although Boxer and Stetten (3) reported a reduction in the deposition of newly synthesized fatty acids in rats on a restricted food intake, the full import of lipogenesis in all of the above conditions has not been recognized. In the present investigation, we have studied the conversion of C^{14}-labeled glucose to fatty acids by liver slices prepared from fasted rats and from rats that had been fed various diets. A failure in hepatic lipogenesis is shown here to be a prominent defect in the fasted animal and in the animal deprived of carbohydrate.

EXPERIMENTAL

Preparation of Liver Slices and Incubation Procedure—The rats were sacrificed by cervical fracture. Their livers were excised and sliced freehand with a razor blade. The slices were collected in a Petri dish containing cold Ringer-bicarbonate solution prepared according to Krebs and Henseleit (4). Approximately 300 mg. of slices were gently blotted on a moist filter paper, weighed on a torsion balance, and placed in a specially designed incubation flask containing 5 cc. of the medium.

The incubation flask used has been described by Chernick *et al.* (5).

It contains a large compartment in which the buffer solution, substrate, and tissue slices are placed, a small center well capped at the bottom with a rubber policeman through which KOH is injected at the end of the experiment, and a side arm which contains the acid.

4.5 cc. of the Ringer-bicarbonate solution and 0.5 cc. of a glucose solution containing C^{14}-glucose were placed in the main chamber of the flask. The concentration of glucose of the medium was approximately 400 mg. per cent. In the side arm was placed 0.25 cc. of 5 N H_2SO_4. A roll of No. 50 Whatman filter paper was snugly fitted into the center well. The flask was aerated with a gas mixture of 95 per cent O_2-5 per cent CO_2, tightly stoppered, and placed in a constant temperature bath of 37.5°. The flask was shaken gently for the 3 hour period of incubation. About 5 minutes before the end of the experiment, 1 to 1.25 cc. of 30 per cent KOH were injected through the rubber policeman into the center well with the aid of a syringe and a No. 27 gage needle. The KOH rapidly distributed itself over the rolled filter paper and served to absorb the CO_2 from the atmosphere of the flask. The experiment was terminated by tilting the flask so that the acid from the side arm was transferred to the main compartment. 15 to 30 minutes were found adequate for complete absorption of CO_2.

The radioactive glucose was prepared photosynthetically according to the method of Putman et al. (6). It was shown to be uniformly labeled with C^{14}. We are indebted to Mr. S. Abraham and Dr. W. Z. Hassid for the preparation of the sample used in this study.

Methods of Analyses

Determination of $C^{14}O_2$ Evolved[1]—The filter paper was removed from the center well of the incubation flask and placed in a 50 cc. volumetric flask. The center well was washed five times with 3 cc. of water, and the washings were transferred to the same volumetric flask, which was then made to volume. An aliquot, usually 5 cc., was removed, and to it was added, as carrier, 1 cc. of 0.25 N Na_2CO_3. An excess of $BaCl_2$ was then added to this mixture, and the $BaCO_3$ was precipitated and mounted on filter paper, as described by Entenman et al. (7).

Determination of Fatty Acids[1]—The acidified contents of the flasks (liver slices and suspending medium) were left in a refrigerator at 4° for 12 hours. The entire contents of each flask were filtered through No. 50 Whatman paper. This separated the *water-soluble material* (Fraction A) from the liver slices and the lipides, which remained on the fil-

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[1] A $C^{14}O_2$ analysis was carried out for each flask. In the case of fatty acids, the liver slices which had been prepared from a single liver and incubated in three flasks were combined for analysis.

ter paper. The contents of the filter paper were exhaustively washed with cold water and these washings were added to the water phase.

The liver slices were picked off the filter paper with a forceps, and placed in an Erlenmeyer flask. A hole was then made at the bottom of the filter paper and the residue on the filter paper was washed into the Erlenmeyer flask with 10 cc. of hot ethyl alcohol. 8 cc. of water and 2 cc. of 90 per cent KOH were added to yield a 50 per cent alcohol solution. The Erlenmeyer flask was placed on a steam bath and refluxed for 12 hours to insure saponification. At the end of that time, the ethyl alcohol was allowed to evaporate. Water and ethyl alcohol were then added to the alkaline hydrolysate until a 50 per cent aqueous alcoholic solution was obtained. This solution was then extracted three times, with large amounts of petroleum ether, in a separatory funnel. The petroleum ether extracts were combined and washed with three 15 cc. portions of 2 N KOH. They were next washed four times with distilled water. These KOH and water washings were then added to the alcohol-aqueous phase which contained the fatty acids in the form of soaps. The petroleum ether extract, which contained the unsaponifiable material, contained little or no radioactivity and was discarded.

The alcohol was removed from the alkaline hydrolysate (the alcohol-aqueous phase referred to above) by allowing it to evaporate on the steam bath. The hydrolysate was then made acid to brom cresol green. This acidified solution was left for 12 hours in the refrigerator, where the fatty acids formed large aggregates. The hydrolysate was filtered through No. 1 Whatman paper and the fatty acids were collected on the filter paper. The filter paper was folded and placed in a Soxhlet flask, where it was extracted for 24 hours with ethyl ether. An aliquot of the ethyl ether extract was evaporated to dryness in a tared flask, and the weight of the fatty acids was determined. Another aliquot was dried and oxidized as described below.

Determination of Total C^{14} in Water-Soluble Material (Fraction A)— The *water-soluble fraction* and the *washings* were made to volume. A protein-free aliquot was prepared with $ZnSO_4$ and NaOH as described by Somogyi (8) and was dried at 60° under reduced pressure. Its C^{14} was determined as described below. The value so obtained is referred to as Residue A in the 3rd column of Table I.

Determination of C^{14} in Compounds Or Fractions—The fatty acids and Residue A were oxidized by being heated with a chromic-sulfuric acid mixture, according to Van Slyke and Folch (9). The CO_2 evolved was trapped in a mixture of 0.25 N $Ba(OH)_2$ and 2 per cent $BaCl_2$. The $BaCO_3$ so formed was mounted on filter paper and counted as described by Entenman *et al.* (7).

Determination of Carbohydrate Content of Liver Slices before Incubation—500 mg. of liver slices were removed from the Petri dish and treated with 5 cc. of 1 N HCl for 3 hours on a steam bath. The volume was kept constant by covering the flask with a bubble stopper. The mixture was neutralized by the addition of solid Na_2CO_3 and transferred to a volumetric flask. An aliquot was treated with $ZnSO_4$ and NaOH after the method of Somogyi (8), the mixture was centrifuged, and the clear supernatant analyzed for glucose (10, 11).

Extent to Which C^{14} Was Recovered in Various Fractions—The major part of the added C^{14} was recovered in the fraction designated above as Residue A. Table I shows that the C^{14} recovered in this fraction, plus

TABLE I

C^{14} *Accounted for by Experimental Determinations (Typical Values)*

Rat No.	Per cent of C^{14} recovered in fatty acids and CO_2	Per cent of C^{14} recovered in Residue A*	Total per cent of C^{14} recovered
3, Table II	5	85	90
5, " II	5	89	94
8, " II	2	94	96
10, " II	1	100	101
3, " V	7	86	93
5, " V	5	94	99
9, " V	3	94	97
10, " V	1	94	95
12, " V	2	92	94
15, " V	2	95	97

* See the text for an explanation.

the C^{14} recovered as fatty acids and CO_2, accounted for 90 to 101 per cent of the added glucose-C^{14}.

Results

Effect of Fasting on Conversion of Added Glucose-C^{14} to Fatty Acids by Surviving Liver Slices

The eleven rats used in this experiment were fed, for 2 weeks, a diet composed of 60 per cent dextrose, 22 per cent casein,[2] 6 per cent brewers' yeast, 6 per cent salt mixture (12), and 6 per cent Cellu flour.[3] Approximately 300 mg. of liver slices were incubated in each flask, and enough slices were prepared, from the liver of each rat, for three such flasks. The

[2] Labco, vitamin-free.
[3] Obtained from the Chicago Dietetic Supply House, Inc.

initial concentration of glucose in the medium was about 400 mg. per cent. The results are shown in Table II.

The CO_2 values are averages of three separate determinations,[1] a single one on each flask. The remaining contents of the three flasks were pooled for the other analyses.

TABLE II

Effect of Fasting on Lipogenesis of Surviving Liver Slices

Rat No.	Weight when sacrificed	Period fasted	Liver slices contained in 3 baths*			Glucose content of medium	Per cent of added glucose-C^{14} recovered in	
			Amount wet weight	Fatty acid content‡	Total carbohydrate content		CO_2†	Fatty acids†
	gm.	*hrs.*	*mg.*	*per cent*	*per cent*	*mg. per cent*		
1	260	Not fasted	905	2.4	4.0	382	3.1	0.54
2	195	" "	930	3.5	3.6	382	4.4	0.72
3	125	" "	982	3.6	3.9	382	3.3	1.32
4	140	24	990	1.3	1.0	362	5.0	0.07
5	120	24	960	1.3	2.5	362	4.1	0.04
6	220	24	945	3.5	1.5	410	2.5	0.07
7	140	48	920	5.3	0.9	362	1.4	0.04
8	120	48	999	5.3	1.0	362	2.3	0.03
9	240	48	935	7.1	1.6	410	0.6	0.02
10	260	72	918	5.4	1.9	410	0.8	0.02
11	240	72	931	5.5	1.9	410	1.3	0.00

* All the slices prepared from a single liver were collected in a Petri dish containing the Ringer-bicarbonate medium. Approximately 300 mg. of these slices were transferred to each of the three flasks. A separate portion, about 500 mg. of slices, was used for determination of total carbohydrate.

† $C^{14}O_2$ collected in each flask was separately analyzed. Hence the value for $C^{14}O_2$ is the average of three separate determinations. In the case of fatty acids, the entire contents of three flasks were combined for a single analysis.

‡ Determined on the whole flask contents at the end of the experiment.

Rats 1, 2, and 3 were sacrificed without being fasted. In 3 hours, about 3 to 4 per cent of the glucose-C^{14} was oxidized to CO_2 by 900 to 980 mg. of liver slices, and about 0.5 to 1.3 per cent was recovered as fatty acids.

The next three rats (Rats 4, 5, and 6) were fasted for 24 hours before slices were prepared from their livers. From 2.5 to 5 per cent of the glucose-C^{14} was converted to CO_2, but only 0.04 to 0.07 per cent was recovered as fatty acids.

With more extensive fasting (48 to 72 hours), the amount of C^{14} re-

covered as fatty acids was even lower. The $C^{14}O_2$ recovered was also reduced below the values observed for either the fed rats or the rats fasted 24 hours.

Rôle of Previous Intake of Carbohydrate, Protein, and Fat on Conversion of Added Glucose-C^{14} to Fatty Acids and CO_2

Having shown that the fate of the glucose is greatly changed by fasting, we became interested in studying the relative influences of the major dietary constituents upon glucose metabolism. The yeast used in the experiment described above was replaced by a synthetic vitamin B mixture.[4] The composition of the diets is described in Table III.

TABLE III

Composition of Diets

All values refer to gm. per 100 gm. of diet.*

Diet	Casein†	Dextrose	Butter	Salt	Cellu flour
A	22	60	0	6	11.8
B	0	82	0	6	11.8
C	82	0	0	6	11.8
D	22	0	60	6	11.8

* Each 100 gm. of diet contained, in addition, the following: 100 mg. of choline chloride, 5 mg. of niacinamide, 100 mg. of inositol, 5 mg. of calcium pantothenate, 1 mg. of thiamine hydrochloride, 1 mg. of riboflavin, 1 mg. of pyridoxine hydrochloride, 1 mg. of p-aminobenzoic acid, 0.02 mg. of biotin. Each rat also received every other day 2 drops of a fish oil containing 400 units of vitamin D and 3000 units of vitamin A per cc.

† Labco, vitamin-free, obtained from The Borden Company.

The caloric intakes and weight changes observed in the rats fed these diets are recorded in Table IV. The results of the experiment are shown in Table V.

Diet A is essentially a high carbohydrate diet containing adequate protein. Each of the rats fed this diet ingested approximately 50 calories per day. It was fed to Rats 1 to 5 for 3 days and to Rats 6 and 7 for 10 days, before slices were prepared from their livers. As in the preceding experiments, approximately 950 mg. of slices were used in each determination. With this amount of slices, about 0.5 to 2 per cent of the added C^{14} was recovered as fatty acids, and from 6 to 9 per cent as CO_2.

The sole source of calories in *Diet B* was carbohydrate. Each animal ingested about 40 calories per day, but lost about 2 gm. of body weight per day. This loss is far less than that which occurs during fasting.

[4] See foot-note, Table III.

Despite the loss of weight observed in these rats, the feeding of Diet B for 3 days did not interfere with the conversion of the added glucose-C^{14} to CO_2 and fatty acids. The results obtained with Diet A were used as standards for comparison in this section.

TABLE IV

Caloric Intake and Weight Changes in Rats Fed Various Diets

Rat No.	Diet fed	Period fed	Total caloric intake in period fed	Weight change by end of period
		days		*gm.*
1	A	3		8
			324*	
2	"	3		12
3	"	3	129	9
4	"	3		15
			310	
5	"	3		21
6	"	10		21
			1050	
7	"	10		18
8	B	3		−5
			230	
9	"	3		−7
10	"	10		−23
			810	
11	"	10		−22
12	C	3		12
			234	
13	"	3		−3
14	"	3		6
			250	
15	"	3		8
16	"	3		0
17	"	3		8
18	"	10		15
			730	
19	"	10		15
20	D	3		12
			324	
21	"	3		11

* With the exception of Rat 3, the food intake was measured for pairs of rats.

The feeding of Diet B for 10 days, however, reduced the amount of C^{14}-glucose that was converted to fatty acids. In none of the animals fed this diet for 3 or 10 days was there a reduction in fatty acid-C^{14} recovery so pronounced as that observed with liver slices of fasted rats.

Diet C, which was fed to Rats 12 to 17 for 3 days and to Rats 18 and 19 for 10 days, contained only protein as the source of calories. Each rat ingested about 40 calories per day and maintained its body weight. In all eight rats, the recovery of C^{14} as fatty acids was greatly reduced. The values for $C^{14}O_2$ were also low as compared with the results obtained with Diet A.

TABLE V

Effect of Previous Diet on Lipogenic Capacity of Surviving Liver Slices

Rat No.	Weight when sacrificed	Diet fed		Liver slices contained in 3 baths*			Glucose content of medium	Per cent added glucose-C^{14} recovered in	
				Wet weight	Fatty acid content*	Total carbohydrate content		CO_2*	Fatty acids*
	gm.		*days*	*mg.*	*per cent*	*per cent*	*mg. per cent*		
1	134	A	3	951	3.5	5.3	402	7.1	1.9
2	146	"	3	945	2.9	4.7	402	9.4	2.3
3	138	"	3	969	3.4	2.3	415	6.0	0.80
4	150	"	3	942	3.2	5.0	406	5.9	0.44
5	150	"	3	921	4.0	3.3	406	6.4	0.84
6	164	"	10	895	3.3	4.5	402	6.3	1.6
7	145	"	10	936	3.2	4.6	402	4.2	0.80
8	98	B	3	924	3.3	10.0	402	5.8	1.6
9	112	"	3	933	2.7	6.6	402	6.0	0.83
10	139	"	10	918	3.3	5.4	402	4.1	0.40
11	122	"	10	954	4.4	6.1	402	3.0	0.19
12	132	C	3	937	2.8	3.2	402	1.4	0.02
13	138	"	3	928	2.8	6.5	402	4.1	0.11
14	138	"	3	949	2.9	3.0	406	1.9	0.03
15	176	"	3	983	2.6	3.5	406	2.0	0.06
16	150	"	3	964	2.9	3.7	406	2.4	0.10
17	130	"	3	930	4.5	4.6	406	2.2	0.10
18	137	"	10	932	2.8	5.1	402	1.7	0.03
19	137	"	10	926	2.9	4.9	402	2.3	0.12
20	144	D	3	954	4.2	1.5	415	1.6	0.03
21	127	"	3	983	6.5	1.9	415	2.2	0.03

* See respective foot-notes, Table II.

The bulk of the calories in *Diet D* was derived from washed butter, but this diet also contained an adequate amount of protein. Rats 20 and 21 were fed this diet for 3 days. These rats each ingested about 54 calories per day. Livers from these rats practically lost their capacity for converting the glucose-C^{14} to fatty acids. Formation of $C^{14}O_2$ was also depressed, but not to the same extent as the fatty acid formation.

DISCUSSION

In the present investigation we have studied the conversion of added glucose-C^{14} to fatty acids and CO_2 by liver slices prepared from fasted rats and from rats that had been fed diets with the following caloric composition: Diet A, 74 per cent carbohydrate and 26 per cent protein; Diet B, 100 per cent carbohydrate; Diet C, 100 per cent protein; Diet D, 86 per cent fat and 14 per cent protein. In the fasted rats and in the rats fed diets devoid of carbohydrate (Diets C and D), the ability of their livers to convert the glucose-C^{14} to fatty acids was greatly depressed or practically absent. This change in the pattern of glucose utilization in the liver is not the result of a severe insult to the animal, for the change was observed after 1 day of fasting and after 3 days of carbohydrate deprivation. The importance of carbohydrate lack in the diet as the causa-

TABLE VI

Showing Lack of Proportionality in Extent of Conversion of Glucose-C^{14} to Fatty Acids and CO_2 by Livers of Normal and Fasted Rats

The mean $C^{14}O_2$ and fatty acid-C^{14} recoveries for the various groups shown in Table II were calculated. All the values recorded below are percentages of the mean value obtained for the unfasted control (0 hour fast).

C^{14} fraction	Period fasted			
	0 hr.	24 hrs.	48 hrs.	72 hrs.
CO_2	100	108	40	30
Fatty acids	100	7	3	1

tive agent in this change is further emphasized by the finding that the livers of rats fed for 3 days a diet composed entirely of carbohydrate as its caloric source showed no impairment in the conversion of the C^{14}-glucose to fatty acids.

In Tables VI and VII we have compared the orders of magnitude in the recoveries of $C^{14}O_2$ and fatty acid-C^{14} in the various nutritional states. It is of interest that, when a decrease in the fatty acid-C^{14} recovery occurred, the $C^{14}O_2$ recoveries were not proportionally decreased. The most interesting case was observed in the rats fasted 24 hours in which the rate of $C^{14}O_2$ formation was not altered at all, whereas the recovery of fatty acid-C^{14} was decreased by 93 per cent. In every case, the extent of decrease in the recovery of fatty acid-C^{14} greatly exceeded that of $C^{14}O_2$. This dissimilarity provides strong evidence that a true defect in lipogenesis occurs in the livers of rats that are fasted or fed a diet devoid of carbohydrate.

The $C^{14}O_2$ recoveries were depressed when the rats were fasted or fed

diets containing no carbohydrate. The amount of $C^{14}O_2$ recovered in an experiment of this kind is a function of (a) the rate of CO_2 formation, (b) the different specific activities of the various immediate precursors of CO_2 at the exact site of reaction, and (c) the percentages of the total CO_2 derived from each of these various precursors. The factors that could influence isotopic recoveries in slice experiments such as those carried out here have been discussed (13–15). Since it is hardly feasible to measure the specific activity of the carbohydrate precursor of CO_2 at the site of the precursor reaction, especially when the rate is unknown, it may be argued that the results presented here do not necessarily indicate that glucose oxidation within the liver cell was depressed. Furthermore, it is not known how fasting or carbohydrate lack affect the permeability of the liver cell, glucqneogenesis from protein, mobilization of glycogen,

TABLE VII

Showing Lack of Proportionality in Extent of Glucose-C^{14} to Fatty Acids in CO_2 in Livers of Rats Fed Various Diets

The mean $C^{14}O_2$ and fatty acid-C^{14} recoveries for the various groups shown in Table V were calculated. All the values recorded below are percentages of the mean value obtained for the control group fed Diet A.

C^{14} fraction	Diet A	Diet B		Diet C		Diet D
	Control	3 days	10 days	3 days	10 days	3 days
$C^{14}O_2$	100	91	55	35	31	29
Fatty acids	100	98	25	5	6	2

etc., factors that undoubtedly influence the specific activity of the various carbohydrate intermediates which determine the amount of C^{14} recovered.

Another factor that influences the recoveries of $C^{14}O_2$ is the amount of carbohydrate present inside the slice at the time the liver was excised from the animal. This initial carbohydrate dilutes the specific activity of the glucose added to the bath and is perhaps the major factor determining the specific activity of the carbohydrate intermediates. It is of interest that the recoveries of $C^{14}O_2$ were lowest in the case of the rats fasted or fed a high fat diet in which, presumably, the dilution of the added C^{14}-glucose was least. Again, the recoveries were the highest when the dilution was the greatest. For example, one of the highest recoveries of $C^{14}O_2$ and fatty acid-C^{14} was observed in slices prepared from Rat 8, even though its liver contained about 10 per cent total carbohydrate, a value twice that observed in the normal liver. The recoveries of $C^{14}O_2$ were least in the fasted and fat-fed animals whose livers contained less than 2 per cent total carbohydrate; *i.e.*, about 50 per cent of the total carbohydrate content of the normal liver. It is therefore probable that the

actual differences in the conversion of glucose to CO_2 by the livers of rats maintained under the various nutritional states described above are more pronounced than are indicated by the $C^{14}O_2$ recoveries.

The results presented here established clearly the importance of ingested carbohydrate in maintaining the liver's capacity to convert glucose to fatty acids. Rats were fed a diet totally devoid of protein, but containing an abundant amount of carbohydrate; yet their livers converted carbohydrate to fatty acids at an apparently normal rate. On the other hand, the feeding of a diet containing protein, but totally devoid of carbohydrate, greatly reduced the rate of this conversion in the liver.

It should not be inferred, of course, that dietary protein is of no importance in lipogenesis. Miller has reported that the liver of the fasted rat loses a variety of enzymes (16). A loss of liver proteins (for example, the loss of a protein enzyme) might, therefore, explain the reduction in hepatic lipogenesis observed in rats that were fed no protein (Diet B) for 10 days.

The liver of the *fasted* rat also loses its lipogenic capacity, and this was found to occur as early as 24 hours after all food was withdrawn. Whether this results from carbohydrate deprivation or from loss of liver protein remains to be investigated.

That lipogenesis does occur in the liver of growing rats while they are fed a diet high in carbohydrate and adequate in protein is not an unexpected finding. But that it should occur in the liver of a rat while a net loss in body weight takes place is surprising. This is the case of the rats fed Diet B in which the sole source of calories was glucose.

How does dietary carbohydrate influence the liver's ability to convert glucose to fatty acid? Obviously, the rôle of insulin must be considered here, particularly in view of Stetten and Boxer's finding that the depression in lipogenesis is a major defect in the alloxan-diabetic rat (17). It has also been stated that the effect of diet on the insulin content of the pancreas is due in large part to the amount of available carbohydrate in the diet (18). Whether an insulin deficiency will account for the depressed hepatic lipogenesis reported here is, at present, being investigated.

SUMMARY

1. The conversion of C^{14}-labeled glucose to fatty acids and CO_2 was studied in surviving liver slices prepared from rats that had been fasted and from rats fed diets in which the carbohydrate, fat, and protein contents were varied.

2. A diet composed of 60 per cent dextrose and 22 per cent casein was used as a standard. About 1 per cent of the added C^{14} was recovered as fatty acids in experiments carried out with livers of rats fed this diet.

3. Fasting reduced the capacity of the liver to convert glucose to fatty

acids. When all food was withheld for only 24 hours, the recoveries of fatty acid-C^{14} were reduced to values less than one-tenth of those found in experiments with the standard diet. The recovery of $C^{14}O_2$ was not affected by the 24 hour fast.

4. In rats fed diets devoid of carbohydrate, hepatic lipogenesis fell off rapidly. A 3 day feeding of a diet composed entirely of protein or of protein and fat resulted in an extraordinary reduction in hepatic lipogenesis.

5. The livers of rats fed, for 3 days, a diet in which carbohydrate was the sole source of calories showed no impairment in their lipogenic capacity. The feeding of the diet for 10 days, however, did moderately reduce the lipogenic activity of the liver.

6. The conclusion is drawn that dietary carbohydrate is essential for maintenance of the capacity of hepatic tissue to convert glucose to fatty acids.

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THE UPTAKE OF RADIOCALCIUM BY THE SKELETON: THE EFFECT OF VITAMIN D AND CALCIUM INTAKE*

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The rates of uptake of the radioisotopes of phosphorus, strontium, and calcium by the bones of experimental animals have been studied under a variety of conditions, and attempts have been made to interpret these data in terms of rates of bone formation. Cohn and Greenberg (1) and Greenberg (2) found, for example, that the proportion of administered P^{32}, Sr^{89}, or Ca^{45} deposited in the skeleton during a period of 72 hours following the administration of these isotopes was greater in rachitic rats treated with vitamin D than in untreated rachitic rats. They concluded that vitamin D directly stimulates the processes of calcification, thereby increasing the rate of deposition in bone of the elements studied. Migicovsky and Emslie (3) studied the effect of vitamin D upon the rate of loss from the body of previously incorporated Ca^{45}. In these experiments, Ca^{45} was given to rachitic chicks with deposition of the absorbed Ca^{45} in the skeleton. After administration of radiocalcium was discontinued, half of the animals were treated with vitamin D and the loss in the excreta of the previously retained Ca^{45} was measured in treated and untreated chicks. The total calcium content and the specific activity of the calcium of the excreta were less in the vitamin D-treated than in the untreated chicks. On this basis, it was suggested that vitamin D functioned by inhibiting the solution of calcium from bone. These conclusions concerning the function of vitamin D in bone formation based on studies with radioisotopes imply that the rate of new bone salt deposition or bone salt dissolution can be measured by the rate of uptake or loss of the radioactive isotope. There is considerable evidence, however, that, when a solid crystalline material is equilibrated with a solution containing a radioisotope of one of the elements forming part of the solid phase, the radioisotope is incorporated into the crystal without any actual change in mass of the solid phase as the result of an exchange of normal isotope of the

solid phase for radioisotope from the liquid phase. This type of exchange has been reported following equilibration of bone either *in vivo* or *in vitro* with solutions containing P^{32} (4, 5). If this rate of exchange is rapid, any conclusions concerning the rate of bone salt deposition based solely on the amount of radioactivity measured in the skeleton after administration of P^{32} or Ca^{45} would be invalid.

In the present experiments, the effect of vitamin D upon the uptake of Ca^{45} by the skeleton of the rachitic rat was studied. By means of measurements of the specific activities of the serum and skeletal calcium at intervals following administration of Ca^{45}, it was possible to determine whether the uptake of Ca^{45} by the skeleton was a measure of bone salt deposition or the result of an exchange of calcium between solid and liquid phases independent of changes in total bone calcium.

Methods

Rachitic animals were prepared by feeding recently weaned rats of the Sprague-Dawley strain a vitamin D-free, high calcium, low phosphorus diet. This diet, which was suggested by Bessey,[1] produced the characteristic bone lesions of low phosphorus rickets but permitted normal growth of the rats. Following a period of 3 to 4 weeks on the rachitogenic diet, the animals weighed between 100 and 150 gm. and were then used for the Ca^{45} studies. The rats were divided into three groups: untreated rachitic rats, vitamin D-treated rachitic rats, and control rats. The animals of the vitamin D-treated group were given 3000 units of vitamin D_2 72 hours before the administration of radiocalcium. The control rats were given vitamin D prophylactically in a dosage of 500 units per week during the time they received the rachitogenic diet. In addition, some of the rachitic, treated, and control rats were placed on a diet almost free of calcium but otherwise identical with the rachitogenic diet, starting 72 hours before the administration of the radioactive calcium and continuing until the animals were sacrificed. These animals comprise the low calcium series of experiments, whereas the rats kept on the rachitogenic diet until the time of sacrifice form the high calcium series. The calcium content of the rachitogenic diet was 1.2 per cent and that of the low calcium diet 0.03 per cent.

[1] Bessey, O. H., personal communication. The composition of the diet is as follows: casein (Labco vitamin-free) 180 gm., dextrose 250 gm., salt mixture 10 gm., calcium carbonate 30 gm., peanut oil 80 gm., vitamin A concentrate (vitamin D-free) 10,000 units, brewers' yeast (vitamin D-free) 50 gm., cornstarch 450 gm. The salt mixture is made up of $MgCO_3$ 25.0 gm., $MgSO_4$ 16.0 gm., NaCl 69.0 gm., KCl 112.0 gm., $FePO_4 \cdot H_2O$ 20.5 gm., KI 0.08 gm., $MnSO_4$ 0.35 gm., $CuSO_4$ 0.09 gm.

Rats of each group were given by stomach tube 1 cc. of a solution of $Ca^{45}Cl_2$ containing 10 mg. of total Ca and less than 1.0 μc. of Ca^{45}. All administered solutions were prepared from a single batch of $Ca^{45}CO_3$. Groups of rats were anesthetized with sodium pentobarbital at intervals of 2, 4, 24, and 72 hours following the administration of Ca^{45} and blood was drawn from the abdominal aorta. Following exsanguination, the animals were skinned and eviscerated, and the skeleton cleaned as free as possible of fat and heavy muscle masses. The remaining carcass was covered with 2 per cent NaOH solution and the soft tissues digested by refluxing overnight on the steam bath. A slight error may be introduced by contact of the bone with tissue fluids containing radioactive calcium for this period of time, but the amount of calcium in the body fluid of the remaining soft tissue is minute compared with the total amount of calcium in the skeleton. The skeleton was washed several times with distilled water, then dissolved with the aid of normal HCl, and the solution was made up to volume. Aliquots of blood serum and skeleton solution were taken for determination of total calcium and radioactivity. Calcium was determined by the method of Kramer and Tisdall (6). Radioactivity of the calcium was measured after precipitation in centrifuge tubes as calcium oxalate. The calcium oxalate was separated by centrifugation, was washed once with 2 per cent NH_4OH, then was suspended in 0.5 cc. of water, and quantitatively transferred with a capillary pipette to ¼ ounce ointment tins, with three washings of 0.5 cc. each. Uniform spreading of the precipitate was obtained by addition of a few drops of a dilute solution of a surface-active agent, Tergitol. The precipitate was dried under an infra-red heater and the radioactivity of the dried film was measured by means of a thin window Geiger-Müller counter tube and a Tracerlab autoscaler counter. The sample was always placed in a fixed position at a constant distance from the window of the counting tube, and the uniformity of the geometry of the measurements, as well as the uniformity of the counting circuit, was checked with a standard β-ray source. The total calcium of the precipitate never exceeded 1.6 mg. and, when necessary, carrier calcium was added to the solution before precipitation of the calcium oxalate so that at least 0.4 mg. of calcium was present. Preliminary experiments were made in which a constant volume of a known solution containing radioactive calcium was added to each of a series of tubes and amounts of calcium chloride solution added so that the total amount of calcium per tube ranged from 0.02 to 1.6 mg. The calcium was precipitated as the oxalate and the precipitate transferred and the radioactivity measured as described above. The results of such an experiment given in Table I indicate that quantitative transfer of the

Measurements of Radioactivity in Calcium Oxalate Precipitated from Solution Containing Constant Amount of Ca⁴⁵ and Variable Quantities of Carrier Calcium

Total carrier Ca in sample	Radioactivity of ppt.
mg.	*c.p.s.*
0.02	21.4
0.34	22.1
0.64	22.3
0.96	22.2
1.28	21.3
1.62	20.1

An amount of $Ca^{45}Cl_2$ solution with a radioactivity of 21.9 c.p.s. was added to each tube.
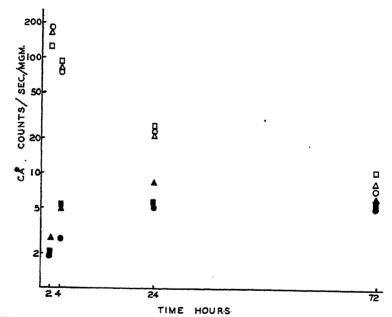
FIG. 1. Comparison of specific activities of serum and skeletal calcium of rachitic, treated, and control rats at intervals following administration of $Ca^{45}Cl_2$. Serum Ca in (△) control rats, (□) treated rats, and (○) rachitic rats; skeletal Ca in (▲) control rats, (■) treated rats, and (●) rachitic rats.

precipitate was obtained and that there was no measurable error due to variation in self-absorption except possibly with the largest amount of carrier calcium added.

The specific activities of the calcium of serum and skeleton are expressed as counts per second per mg. of calcium. These values are all corrected for radioactive decay to the same arbitrary reference time.

Results

The relationship between the specific activities of the calcium of serum and total skeleton for the three groups of rats at intervals following administration of $Ca^{45}Cl_2$ solution is shown graphically in Fig. 1. Each point is the average of the values obtained in a group of three to eight animals sacrificed at the time noted along the abscissa. For convenience,
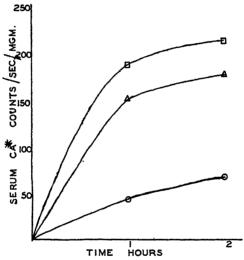
FIG. 2. Specific activities of serum calcium of individual rats during first 2 hours after administration of $Ca^{45}Cl_2$. ($\triangle$) control rat, ($\square$) treated rat, ($\bigcirc$) rachitic rat.

the specific activities are plotted on a logarithmic scale. The curves for uptake of Ca^{45} by the skeleton and disappearance of Ca^{45} from the serum are essentially the same for the rachitic, treated, and control series of rats.

In Fig. 2 are shown three curves representing the specific activities of the serum calcium of three individual animals during the first 2 hours after administration of Ca^{45}. Blood samples were taken from the tail and serum calcium determined by the microtechnique of Sobel and Sobel (7). The average specific activity of the serum calcium during this 2 hour period can be calculated from the area under the curve. The total radioactivity of the skeleton was determined at the end of the 2 hour

period. If the uptake of radiocalcium by the skeleton were entirely due to deposition of new bone salt, the amount of calcium deposited in the skeleton during this time would be obtained by dividing the total radioactivity of the skeleton by the average specific activity of the serum calcium during the 2 hours. Such calculations for each of the three experiments illustrated in Fig. 2 indicate that the addition of approximately 20 mg. of calcium to the skeleton would be necessary to account for the measured radioactivity on the basis of new formation. The total absorption of administered calcium during the 2 hour period was determined in these same experiments and found to range from 1 to 4 mg. Assuming that the concentration of body fluid calcium was not increased and urinary excretion was negligible so that the amount of calcium deposited in the skeleton was equal to that absorbed, the available calcium is only a small fraction of the value of 20 mg. calculated above. It is evident that the greater part of the radioactivity of the skeleton cannot be accounted for by increase of the calcium content of the skeleton but must represent an exchange of Ca^{40} from the skeleton for Ca^{45} of the body fluids. This exchange might be the result of two processes: dissolution of bone occurring simultaneously with formation of new bone, or atomic exchange of calcium between crystalline phase and the liquid phase without change in the actual crystal structure. This second type of reaction has been demonstrated *in vitro* (5) when bone is equilibrated with phosphate solutions containing P^{32}.

Knowledge of the rate of exchange between calcium of body fluids and calcium of the solid phase of bone would be of considerable value. Under the conditions of the present experiments the specific activity of the serum calcium rises to a maximum rapidly and then progressively decreases. As the specific activity of calcium in the body fluids falls, the fraction of calcium of the bone salt which initially exchanged with the body fluid calcium will have a higher specific activity than the calcium of the serum. Simultaneously with continued exchange of Ca^{40} of bone for Ca^{45} of serum, exchange of Ca^{45} in the reverse direction will occur; so that the specific activity of the portion of the bone calcium which has equilibrated with the calcium of the body fluids will parallel the specific activity of serum calcium with some degree of lag. This lag cannot be estimated from available data. If the lag is slight, as seems probable, the rate of exchange of calcium can be measured by comparison of the specific activities of the calcium of the total skeleton and the calcium of the serum. The ratio of these specific activities at a given time indicates the proportion of total skeletal calcium which has exchanged with the body fluid calcium during the interval between administration of the radioactive calcium and the time at which the samples were obtained.

The ratios of specific activity of skeletal calcium to specific activity of serum calcium were calculated for the individual rats of all of the groups and the mean values for each group are given in Table II. The number of animals in each group is indicated by the figures in parentheses. The animals of the high calcium series were fed a high calcium diet throughout, and the rats of the low calcium series were given a low calcium diet from 72 hours preceding the administration of $Ca^{45}Cl_2$ up to the time of sacrifice. The slight differences between the two series are within the range of variability of each group. The data for these two series were combined therefore for purposes of comparison of the ratios of the rachitic, control, and treated groups. At the 24 and 72 hour intervals, the mean

TABLE II

Comparison of Changes in Ratio of Specific Activity of Skeletal Calcium to Specific Activity of Serum Calcium

Group	Time after $Ca^{45}Cl_2$ administration			
	2 hrs.	4 hrs.	24 hrs.	72 hrs.
Rachitic, high Ca..	0.022 (3)	0.049 (10)	0.237 (7)	0.566 (5)
" low " ..		0.078 (1)	0.218 (4)	0.443 (3)
Control, high " ..	0.020 (3)	0.039 (5)	0.239 (6)	0.756 (5)
" low " ..		0.059 (2)	0.182 (2)	0.610 (1)
Treated, high " ..	0.028 (3)	0.057 (8)	0.307 (7)	0.767 (4)
" low " ..			0.325 (2)	0.676 (2)
All rachitic........		0.050 ± 0.014*	0.230 ± 0.059	0.520 ± 0.140
" control........		0.044 ± 0.017	0.224 ± 0.042	0.732 ± 0.124
" treated........		0.057 ± 0.016	0.312 ± 0.052	0.737 ± 0.150

The values listed are the mean ratios for each group with the number of animals in the group in parentheses.

* Mean ± standard deviation.

values of the ratio of skeletal to serum specific activity for the rachitic group are less than those of the treated groups, and the differences are statistically significant (24 hours, $t = 3.25$, $n = 18$, $p = 0.01$; 72 hours, $t = 2.78$, $n = 12$, $p = 0.02$). Although no difference at 24 hours is found between rachitic and control rats, the specific activity ratio at the 72 hour interval is greater in the control group ($t = 2.61$, $n = 11$, $p < 0.05$). In Fig. 3 the mean ratios of skeletal to serum specific activity for each of the three groups are plotted against time, with use of logarithmic scales for both ordinate and abscissa. There is an approximately linear relationship between the logarithms of the specific activity ratio and time. The time at which the specific activity of total skeletal calcium is 50 per cent of that of the serum calcium can be estimated from the data of Fig. 3,
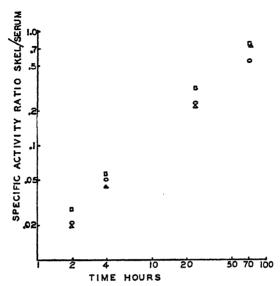
Fig. 3. Relationship of the specific activity ratios of skeletal and serum calcium to time elapsed between administration of Ca⁴⁵ and sacrifice of the animal. The points represent the means for the groups of rachitic, control, and treated rats. (O) rachitic group, (□) treated group, (△) control group.

TABLE III

Specific Activities of Calcium of Serum, Epiphyseal Bone, Diaphyseal Bone, and Total Skeleton at Intervals Following Administration of $Ca^{45}Cl_2$

Time	Group	Serum	Epiphyseal bone	Diaphyseal bone	Total skeleton	$\dfrac{\text{Epiphyseal}}{\text{Diaphyseal}}$
2 hrs.	Rachitic	68	2.4	0.9	1.2	2.7
	Control	175	5.4	2.1	3.6	2.6
	Treated	212	10.8	2.7	5.4	4.0
4 "	Rachitic	83	4.2	1.6	3.0	2.6
	Control	87	3.1	1.0	2.4	3.1
	Treated	72	8.2	2.5	5.3	3.3
72 "	Rachitic	9.3	7.8	3.6	5.2	2.2
	Control	7.8	7.6	3.8	5.3	2.0
	Treated	10.1	9.4	4.3	7.1	2.2
2 wks.	Rachitic	2.1	2.5	2.5	2.5	1.0
	Control	3.1	3.1	2.5	3.2	1.2
	Treated	2.6	2.6	2.4	2.8	1.1

The values are expressed as counts per second per mg. of Ca. The data for the 72 hour periods are averages of determinations in three animals of each group. The other data represent determinations in single rats.

and the value so obtained is approximately 45 hours for the treated group and approximately 65 hours for the rachitic group.

The rate of exchange of calcium is not uniform throughout the various portions of the skeleton. In Table III are shown the specific activities of the calcium of the epiphyseal ends of the long bones and the diaphyseal portions of these bones, and the average values for total skeletal calcium including the portions separately determined. The ratios of the specific activities of epiphyseal to diaphyseal bone are given in the last column of Table III. The specific activity of epiphyseal bone is higher than that of diaphyseal bone in the early phase. Gradually the values for the various portions of the skeleton approach each other, and 2 weeks after administration of Ca^{45} the specific activities of epiphyseal, diaphyseal, total skeletal, and serum calcium are approximately equal. These findings are consistent with the hypothesis that not only the calcium of the superficial layers of the bone structure but also the calcium of the deeper layers of the bone trabeculae exchange with the calcium of the body fluids until the ratio of Ca^{45} to Ca^{40} becomes uniform throughout the entire skeleton and equal to that of the interstitial body fluid. The more rapid equilibration of the epiphyseal ends may be in part due to deposition of and more rapid exchange with the new bone salt at the growing ends of the bone, but local anatomical differences probably also play a rôle. Because of the spongy trabecular structure of this portion of the bone, the interstitial fluid is in contact with a greater surface of bone salt than in the more compact tubular bone of the shafts. In addition, the dissolution and redeposition of bone crystals may occur more rapidly in the actively growing portion of bone.

In proportion to the total mass of skeletal calcium, the rate of exchange of skeletal calcium with body fluid calcium decreases with increasing age and size of the animal. The results reported above were obtained in rats of approximately 6 to 8 weeks of age, weighing 100 to 150 gm. Similar studies were made in a group of twelve animals 15 to 20 weeks of age, weighing 250 to 300 gm. Determinations of the specific radioactivity of serum and skeletal calcium were made 24 hours after administration of $Ca^{45}Cl_2$ solution by stomach tube. At this time the ratio of the specific activity of total skeletal calcium to the specific activity of serum calcium averaged 0.094, in contrast to an average value of 0.257 for the entire series of younger rats.

DISCUSSION

The data presented indicate that the uptake of radioactive calcium by the skeleton following administration of $Ca^{45}Cl_2$ cannot be used as a measure of new bone salt deposition in the period between the absorption of the

Ca[45] and the time at which the determination of the radioactivity of the skeleton is made. Part of the skeletal radioactivity is due to addition of bone salt, but the exchange of calcium between solid and liquid phase is sufficiently rapid so that differences in the rate of bone formation are probably not apparent in the over-all rate of radiocalcium uptake. In these experiments the total Ca[45] content of the skeleton of the treated animals is greater than in the rachitic rats due to more complete absorption of the administered calcium in the animals given vitamin D. This question of intestinal absorption will be discussed in more detail in another paper. It is questionable, however, whether the differences observed between rachitic rats and treated rats with respect to rate of exchange of radiocalcium between serum and skeleton can be explained in terms of rate of bone salt deposition. If this were the explanation, it might be expected that differences would be found between the animals on a high calcium diet and those receiving practically no calcium for as long as 6 days. In each group, however, the data for the low calcium experiments are in approximately the same range as in the high calcium intake experiments. The results suggest that there is an increased rate of turnover of skeletal calcium in the vitamin D-treated rats compared with untreated rachitic animals irrespective of calcium intake.

The differences between rachitic and vitamin D-treated rats are less striking than the fact that in young animals, both rachitic and treated, there is evidence of a rapid rate of exchange between the calcium in the solid phase of bone and the calcium in solution in the body fluids. If the ratio of skeletal to serum specific activity can be used to measure the rate of exchange of calcium between skeletal and body fluids, the time necessary for 50 per cent of the skeletal calcium to exchange with serum calcium is in the range of 45 to 65 hours. Neuman and Riley (4) and Falkenheim, Neuman, and Hodge (5) on the basis of studies of rate of uptake of radiophosphorus by bone both *in vivo* and *in vitro* have previously concluded that exchange of phosphorus between bone and body fluids is necessary to account for the rate of uptake of radiophosphorus by bone. These authors have suggested that only about one-fifth of the total number of phosphorus atoms of the bone crystals can exchange with the phosphorus of the liquid phase. The results of the present *in vivo* studies with Ca[45] indicate that essentially all of the calcium of the bone can exchange with the calcium of the body fluids. The exchange of calcium *in vivo* can presumably be the result both of metabolic dissolution of bone, with simultaneous deposition of new bone salt, and of atomic exchange without actual change of crystal structure. The rate of exchange can therefore be expected to be a function of the metabolic activity of bone, of the anatomical structure with respect to the relationship of bone trabeculae and

interstitial fluid, and of the microcrystalline structure of the bone salt. The relative decrease of the rate of uptake of Ca^{45} with increasing age might be due to differences in bone structure or to lessened metabolic activity or both. Neuman, Neuman, and Mulryan (8) found that the rate of uptake of administered uranium by the bone was more rapid in young rats than in older animals. They suggested that differences in vascularity of bone might be a factor.

SUMMARY

Radioactive calcium was given by stomach tube to rachitic, vitamin D-treated rachitic, and control rats, and groups of animals were sacrificed at intervals of from 2 hours to 2 weeks following administration of Ca^{45}. The radioactivity and total calcium content of blood serum and skeleton were determined, which permitted calculation of the specific activity of serum and skeletal calcium.

Analysis of the data indicates that essentially all of the bone calcium of young rats is rapidly exchangeable with body fluid calcium. The time necessary for 50 per cent of the total skeletal calcium to exchange with body fluid calcium was estimated in 6 to 8 week-old rats to be approximately 45 hours in vitamin D-treated rats and 65 hours in rachitic rats. The rate of turnover of skeletal calcium was apparently increased by administration of vitamin D. In each group, the rate of calcium exchange was not appreciably influenced by changes in dietary calcium intake. The calcium of epiphyseal bone reached equilibrium with serum calcium more rapidly than did that of diaphyseal bone. In proportion to total skeletal calcium, exchange of calcium between skeleton and body fluids was much slower in older rats (15 to 20 weeks) than in 6 to 8 week-old animals.

Because of the rapid exchange of calcium between bone and body fluids, the uptake of Ca^{45} by the skeleton following its administration to the animal cannot be used as a measure of new bone salt deposition.

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SYNTHESIS OF p-AMINOHIPPURIC ACID BY MITOCHONDRIA OF MOUSE LIVER HOMOGENATES

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In studies on the enzyme system in rat liver which synthesizes p-amino-hippuric acid (PAH) from p-aminobenzoic acid (PAB) and glycine, Cohen and McGilvery (1) found most of the enzyme activity in the washed residue of the homogenate. Since their preparation contained all the nuclei and part of the mitochondria of the homogenate, the relative activities of the individual intracellular components with respect to PAH synthesis were thus not clearly defined. The complex nature of the process, which appears to require adenosine triphosphate (ATP) and conditions necessary for oxidative phosphorylation for active synthesis, suggested that the activity may be associated with the mitochondria, wherein reside many of the oxidative enzymes of the tricarboxylic acid cycle (2–9).

Since the fractionation of the homogenate by means of centrifugation, with the KCl solution used by Cohen and McGilvery, does not permit adequate separation of nuclei and mitochondria due to the agglutination of the latter in this medium (3), it was necessary to turn to fractionation methods with sucrose solutions. Preliminary experiments with mouse liver homogenates indicated that PAH synthesis would proceed in isotonic sucrose[1] homogenates at essentially the same rate as in isotonic KCl homogenates. Employing isotonic (0.25 M) sucrose as a medium for the cell fractionation, we have studied the intracellular distribution of PAH synthesis in mouse liver homogenates, with particular emphasis on the synthesis of PAH by mitochondria and some of the conditions responsible for interactions occurring in recombinations of mitochondria with other cell fractions.

EXPERIMENTAL

Preparation of Tissues—Livers from C₃H strain mice were used. The animals were killed by cervical dislocation and the livers (*in situ*) were quickly perfused with ice-cold isotonic sucrose to remove most of the blood. The livers were removed, covered with cracked ice, and, after

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[1] Mitochondria isolated from 30 per cent sucrose (3) and resuspended in either isotonic sucrose or isotonic KCl failed to show any significant synthesis of PAH.

thorough chilling, pressed through a chilled, stainless steel, hand tissue press into a small tared beaker containing a measured quantity of ice-cold isotonic sucrose. The weighed liver tissue was homogenized with 9 volumes of ice-cold isotonic sucrose in an all-glass homogenizer and the homogenate was then passed four times through a No. 20 gage hypodermic needle with a syringe. These operations were carried out in a crushed ice bath at 0°.

20 ml. of the 10 per cent liver homogenate (1 gm. of liver in 10 ml.) were centrifuged for the cell separation according to the method of Schneider (2) with slight modifications. The modifications were recentrifugation of the supernatant after sedimentation of the main bulk of the mitochondria for 10 minutes at 8500 $\times$ g in order to separate an additional small amount of mitochondria and centrifugation of the combined resuspended mitochondria at 18,000 $\times$ g for 10 minutes during the two washing steps. The final supernatant, containing submicroscopic particles and soluble material, was not fractionated. The cell fractions were resuspended in isotonic sucrose in a definite volume and given designations as follows: nuclear fraction, N_W, 10 ml.; mitochondrial fraction, M_W, 10 ml.; and supernatant, S_1, 40 ml. A portion of the supernatant was heated at 100° for 5 minutes and was labeled S_{1B}.

Recombinations of cell fractions were prepared in separate tubes beforehand in order to avoid possible interaction of one cell fraction and the substrate before addition of another cell fraction.

Substrates—Commercial PAB was decolorized with Darco in boiling water and recrystallized from 30 per cent ethanol and from hot water. Commercial PAH was similarly decolorized, recrystallized twice from hot water, and washed with ethanol. The purified PAB and PAH were chromogenically equivalent on a molar basis. Glycine, L-histidine, L-glutamic, α-ketoglutaric, succinic, fumaric, L-malic, and citric acids were commercial products. Potassium pyruvate, oxalacetic acid, and K-d-isocitrate were kindly supplied by Dr. A. Meister and Dr. G. H. Hogeboom. Cytochrome c and the dibarium salt of ATP were obtained from the Sigma Chemical Company, St. Louis, Missouri. The ATP preparation contained at least 90 per cent ATP and less than 2 per cent of the total P was inorganic P. K-ATP solutions were prepared by the method described by Kielley and Meyerhof (10).

Stock media containing substrates 4 times the concentration in the final incubation mixture were prepared. The stock media were adjusted to give, after a 4-fold dilution, pH 7.55 and were kept in glass tubes in a deep freeze. 1 ml. of the stock medium provided the basic substrates for the individual incubations. Other substrate additions were made from solutions adjusted to pH 7.55 with KOH or KHCO$_3$.

Procedure—The enzyme was added to the cold media in 25 ml. Erlen-

meyer flasks kept on shaved ice. After all the enzyme additions were made, the flasks were quickly placed in the Dubnoff metabolic shaking incubator and allowed to equilibrate for 5 minutes with shaking. The flasks were then stoppered and the covers of the incubator put in place. The incubations were carried out at 38° for 55 minutes with shaking at 120 excursions per minute in air as the gas phase. The incubations were terminated by placing the flasks on shaved ice and adding 3 ml. of 0.289 N trichloroacetic acid (TCA). The precipitated proteins were centrifuged and 4 ml. of the TCA filtrate were neutralized with 1 ml. of 0.502 N NaOH. 2 ml. of the neutralized TCA filtrate were used for the PAH determination and appropriate aliquots were taken for P determinations.

Analytical Methods

PAH Determination—The method of Cohen and McGilvery (11, 12) was used.

Phosphorus and Nitrogen—Inorganic P was determined by the Fiske-Subbarow method. Acid-labile P (7′ P) was determined as the difference in inorganic P after hydrolysis in 1 N HCl at 100° for 7 minutes. Total nitrogen was determined by the micro-Kjeldahl procedure, according to Ma and Zuazaga (13).

Results

The intracellular distribution of PAH synthesis in mouse liver homogenates was studied first under conditions similar to those described by Cohen and McGilvery (1) for rat liver homogenates. Preliminary experiments showed that under these conditions, although the mitochondrial fraction accounted for 60 per cent of the total homogenate synthesis, the specific activity of the mitochondria was lower (0.71 μM of PAH per mg. of N) than the values reported by Cohen and McGilvery for the washed residue of rat liver (1.41 to 1.86 μM of PAH per mg. of N). Although ATP had been added to the system, it seemed desirable to determine whether the low activities in our system could be due to rapid loss of ATP during the incubation without adequate concomitant resynthesis. The phosphate buffer in the system was therefore replaced by histidine in order to increase the accuracy of the P determinations. The replacement of phosphate by histidine resulted in even lower specific activities for mitochondria (average, 0.57 μM of PAH per mg. of N), which indicated that phosphate was necessary to obtain higher rates of PAH synthesis. However, it was found that the addition of *boiled* supernatant to mitochondria in the histidine system greatly increased PAH synthesis and the 7′ P levels[2]

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[2] Although there is some question about the nature of the acid-labile P (7′ P) found in systems such as we have studied, there being some evidence of pyrophosphate formation (17, 19), we were unable to demonstrate that inorganic pyrophos-

found after incubation. The activating effect of *boiled* supernatant on PAH synthesis was considerably larger than that which could be expected on the basis of its phosphate content.

Effect of Glutamate—The activating effect of *boiled* supernatant on PAH synthesis by mitochondria suggested that fumarate may not support ATP resynthesis at sufficiently high rates for maximal PAH synthesis. Various intermediates were therefore tested for their effect on PAH synthesis. The results are seen in Table I. Of the various acids tested, glutamate and α-ketoglutarate showed the greatest activating effect, which in the washed residue obtained from the KCl homogenate was 4 to 5 times the effect noted with fumarate alone. The combination of fumarate plus glutamate was slightly more effective than glutamate alone as tested with mitochondria isolated and washed four times in isotonic sucrose. Compared with fumarate alone, this combination generally gave a 2-fold increase in PAH synthesis with the usual twice washed mitochondria.

Cohen and McGilvery (11) considered a possible complication arising from the addition of glutamate to the PAH-synthesizing enzyme system, the formation of p-aminobenzoylglutamic acid which could give false high values for PAH. However, in experiments not reported here, we could find no evidence that such a reaction accounts for the activating effect of glutamate under the conditions employed. When glycine was replaced by glutamate, the amount of conjugated PAB determined in both mitochondria and homogenates by the usual method was negligible and not significantly greater than the controls containing no glutamate or glycine.

The intracellular distribution of PAH synthesis and maintenance of 7' P levels in the presence of fumarate plus glutamate, compared with fumarate alone, is shown in Table II, Experiment 1. PAH synthesis was stimulated by glutamate in all the cell fractions and their recombinations, except the supernatant, which had no significant activity by itself. The activation was particularly striking in the case of mitochondria and mitochondria combined with nuclei, the increase in PAH synthesis being 100 and 200 per cent, respectively, of the synthesis obtained in the presence of

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phate was formed to any appreciable extent. In experiments not reported here, we have found that, whereas nuclei will completely hydrolyze the acid-labile P present in boiled filtrates of mitochondrial incubation mixtures within a short time (the acid-labile P of ATP is similarly hydrolyzed), less than 10 per cent of an equivalent quantity of added inorganic pyrophosphate P was split under the same conditions. These results would indicate that inorganic pyrophosphate formation was probably not significant, at least in the experiments with mitochondria, and that the 7' P found after incubation was derived principally from the adenylic system. Lehninger and Smith (20) reached analogous conclusions with regard to pyrophosphate formation by an enzyme system similar to the one studied here.

fumarate alone. The increase in PAH synthesis in these instances was directly proportional to the increase in $7'$ P levels.

TABLE I

Effect of Various Intermediates on PAH Synthesis by Mitochondrial Preparations from Mouse Liver Homogenates

The flasks contained 1 ml. of stock medium, 0.5 ml. of enzyme equivalent to 100 mg. of fresh liver, 2.1 ml. of isotonic sucrose, 0.0 to 0.4 ml. of 0.1 M intermediate to give the concentrations indicated, in a total volume of 4 ml. (the difference added as water). Final concentrations of substrates (in stock medium) are as follows: 0.001 M PAB, 0.015 M glycine, 0.04 M KCl, 0.05 M histidine, 0.0025 M $MgSO_4$, 0.0005 M ATP, and 1.2×10^{-5} M cytochrome c. Tissue N in Preparation 1, 1.27 mg. per flask; in Preparation 2, 0.91 mg. per flask. The preparations were obtained from different mouse livers.

Preparation No.	Intermediate	PAH formed	$\dfrac{\text{PAH formed}}{\text{Control}}$
		μM	
1. Residue washed 6 times, isotonic from KCl homogenate (1 gm. fresh liver in 10 ml.), centrifuged at 8500 × g 10 min.	Control	0.038	1.0
	0.005 M fumarate	0.203	5.3
	0.01 " "	0.212	5.6
	0.01 " succinate	0.469	12.4
	0.01 " malate	0.263	6.9
	0.01 " citrate	0.349	9.2
	0.01 " isocitrate	0.541	14.3
	0.01 " glutamate	0.895	23.6
	0.01 " α-ketoglu-tarate	0.764	20.1
	0.01 " oxalacetate	0.380	10.0
	0.01 " pyruvate	0.184	4.8
	0.005 " oxalacetate / 0.005 " pyruvate	0.475	12.5
2. Mitochondria isolated in usual manner, washed 4 times with isotonic sucrose	Control	0.130	1.0
	0.0025 M fumarate	0.312	2.4
	0.01 " "	0.350	2.7
	0.0025 " succinate	0.383	2.9
	0.01 " "	0.424	3.3
	0.0025 " glutamate	0.499	3.8
	0.01 " "	0.499	3.8
	0.01 " succinate / 0.005 " glutamate	0.511	3.9
	0.01 " fumarate / 0.005 " glutamate	0.546	4.2

In the presence of fumarate alone, both the supernatant (S_1) and the *boiled* supernatant (S_{1B}) activated PAH synthesis in all the cell fractions and their recombinations. In the presence of added glutamate, however, consistent activation was observed only with the *boiled* supernatant and

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only to a limited extent (10 to 20 per cent). This slight activation was possibly due to inorganic phosphate, since the system did not contain added inorganic phosphate. In the presence of added glutamate, the supernatant exhibited inhibition, slight activation, or no effect on PAH synthesis by mitochondria or mitochondria combined with nuclei. However, maintenance of 7' P levels was always adversely affected by the supernatant under these circumstances. In Experiment 1, Table II, the super-

TABLE II

Intracellular Distribution of PAH Synthesis and Maintenance of 7' P under Various Conditions in Mouse Liver Homogenates

The flasks contained 1 ml. of stock medium, 1 ml. of homogenate (100 mg. of fresh liver) or its equivalent in a larger or smaller volume, and isotonic sucrose to make a final volume of 4 ml. Final concentrations of substrates (in stock medium) are as follows: 0.001 M PAB, 0.015 M glycine, 0.005 M fumarate, 0.005 M glutamate (when added), 0.005 M phosphate (when added), 0.04 M KCl, 0.05 M histidine, 0.0025 M MgSO$_4$, 0.0005 M ATP in Experiment 1 or 0.001 M ATP in Experiment 2, and 1.2×10^{-5} M cytochrome c.

	Experiment 1					Experiment 2				
Cell fractions	Total N	Fumarate		Fumarate + glutamate		Total N	Fumarate + glutamate		Fumarate + glutamate + PO$_4$	
		PAH	7' P	PAH	7' P		PAH	7' P	PAH	7' P
	mg.	μM	γ	μM	γ	mg.	μM	γ	μM	γ
Homogenate.........	2.680	1.133	24	1.295	22	3.262	1.611	253	2.080	323
N$_W$................	0.399	0.002	18	0.043	16	0.410	0.025	55	0.032	94
M$_W$................	0.882	0.448	48	0.934	96	1.022	1.154	283	1.887	305
S$_1$....................	1.357	0.010	6	0.010	8	1.890	0.0		0.0	
N$_W$ + M$_W$...........		0.387	19	1.230	53		1.442	256	2.011	297
M$_W$ + S$_1$.............		0.569	16	0.733	13		1.260	217	1.658	310
" + S$_{1B}$............		0.733	89	1.048	105		1.359	269	1.758	346
N$_W$ + M$_W$ + S$_1$......		0.694	13	0.940	16		1.414	179	1.786	323
" + " + S$_{1B}$.....		0.879	50	1.269	73		1.680	236	1.915	300
			142*		142*			290*		290*

* 7' P added as ATP before incubation.

natant inhibited PAH synthesis by mitochondria, whether tested separately or combined with nuclei. Although PAH synthesis was stimulated by glutamate in these recombinations containing supernatant, there was no corresponding increase in the 7' P levels which were essentially the same low levels as were found in the presence of fumarate alone. The same situation applies to the homogenate, which necessarily contains components of the supernatant. It has frequently been observed that the 7' P levels of homogenates under these conditions fluctuated from relatively high

values approaching those of mitochondria to low values such as are presented in this experiment. The 7′ P levels of recombined cell fractions containing the supernatant, however, were always as low as or lower than the corresponding 7′ P levels of the homogenate.

The variable effects of supernatant on PAH synthesis by mitochondria and mitochondria combined with nuclei are seen by comparing the PAH synthesized in the presence of fumarate and glutamate in Experiments 1 and 2, Table II. In Experiment 1 the supernatant S_1 inhibited PAH synthesis by mitochondria, whereas in Experiment 2 PAH synthesis by mitochondria was slightly enhanced. The supernatant inhibited PAH synthesis by the recombination, $N_W + M_W$, in Experiments 1 and 2, but the degree of inhibition was much greater in Experiment 1. In both experiments, however, the 7′ P levels were significantly reduced when the supernatant was added to either M_W or $N_W + M_W$. Since PAH synthesis was not reduced by *boiled* supernatant and 7′ P levels were maintained at the same or slightly lower levels in the presence of *boiled* supernatant, enzymatic activities which interfere with ATP resynthesis may be present in the supernatant S_1 and account for its inhibitory effect.

Effect of Inorganic Phosphate—The effect of inorganic phosphate on PAH synthesis was already seen in the higher specific activity of mitochondria in phosphate buffer (0.71 μM of PAH per mg. of N) as contrasted with that in histidine buffer (0.57 μM of PAH per mg. of N). The amount of inorganic phosphate necessary for maximal stimulation of PAH synthesis in homogenates and mitochondria was quite small. Maximal stimulation was obtained when 2.5 μM of inorganic phosphate (the smallest quantity tested) was added to each flask. The intracellular distribution of PAH synthesis and 7′ P levels of the homogenate in the system containing added inorganic phosphate in addition to glutamate is seen in Table II, Experiment 2. Comparable values obtained with the same cellular preparations in the absence of added inorganic phosphate are also shown in this experiment.

The results show that inorganic phosphate stimulated PAH synthesis in all the cell fractions and their recombinations. In the presence of added inorganic phosphate and glutamate, the mitochondria accounted for 91 per cent of the total PAH synthesis of the homogenate. The specific activity of mitochondria was 1.85 μM of PAH per mg. of N. 97 per cent of the total homogenate synthesis was recovered in the recombination of mitochondria with nuclei. The 6 per cent increase in PAH synthesis obtained by the recombination probably represents mitochondrial activity in the nuclear fraction.

When no inorganic phosphate was added to the system, the percentage increase in PAH synthesis caused by the addition of nuclei to mitochondria

was significantly greater, 18 per cent, and there was a noticeable decrease in the 7′ P level with a corresponding increase in inorganic phosphate. The activating effect of nuclei on mitochondria in the system containing no added inorganic phosphate therefore appears to be in providing higher inorganic phosphate levels by enzymatic dephosphorylation, which favors higher rates of oxidative phosphorylation and PAH synthesis (7).

The activating effect of the supernatant and of the *boiled* supernatant on PAH synthesis in the system containing no added inorganic phosphate (Experiments 1 and 2, fumarate + glutamate, Table II) completely disappeared when inorganic phosphate was added and, instead, some inhibition, heretofore masked by the larger activating effect, was obtained. It was previously suggested that the inhibition of PAH synthesis in mitochondria by the supernatant, which was also frequently observed, may be associated with some enzymatic activity present in the supernatant which interferes with ATP resynthesis. This suggestion was based on the observation that the inhibition in the system containing no added inorganic phosphate was paralleled by lower 7′ P levels. In the complete system containing added inorganic phosphate, the inhibition of PAH synthesis by the supernatant cannot be related to the 7′ P levels found, which in every case were slightly higher than the 7′ P initially added to the system as ATP. This finding indicates that inhibition of PAH synthesis by the supernatant may be quite complex. In addition to factors which may interfere with ATP resynthesis, which have largely been overcome in the complete system, other factors may be operating to inhibit other parts of the PAH-synthesizing system. The inhibition of PAH synthesis in mitochondria by boiled supernatant also indicates that a stable factor may be involved.

Although the inhibitory nature of the supernatant has not been extensively studied, it would appear from observations on a large number of experiments performed under various conditions that the inhibitory activities associated with the supernatant are probably present in the homogenate and are released during the cell fractionation. Whether or not inhibition is apparent in the homogenate or in the recombined cell fractions depends upon the extent to which inhibiting activities have been developed and the ability of the system to overcome these effects. In the final system containing PAB, glycine, fumarate, glutamate, phosphate, ATP, K^+, Mg^{++}, and cytochrome c, in histidine buffer, the inhibitory effects of the supernatant on PAH synthesis, which had appeared as inhibitions of oxidative phosphorylation in the incomplete systems, have been minimized by increasing the rate of oxidative phosphorylation. The small residual inhibition on PAH synthesis by the supernatant in the complete system prevented complete recovery of all the original homogenate synthesis in

the reconstituted homogenate ($N_W + M_W + S_1$). By omitting the supernatant from this recombination, 97 per cent of the homogenate synthesis was recovered as contrasted with 85 per cent recovery in the presence of supernatant.

A summary of the relative PAH-synthesizing activities of mouse liver homogenates and mitochondria under the various conditions studied is presented in Table III. The specific activities of both homogenates and mitochondria show considerable variation from one preparation to another under a given set of conditions. It is seen that the activating effect of glutamate and inorganic phosphate was more pronounced on mitochondria than on the homogenate. Glutamate caused about a 100 per cent increase

TABLE III

Specific PAH-Synthesizing Activities of Mouse Liver Homogenates and Isolated Mitochondria Studied under Various Conditions

Condition	Tissue	Average specific activity, μM PAH per mg. N	No. of determinations	Range μM PAH per mg. N
Fumarate, PO_4	Homogenate	0.413	5	0.353–0.492
	Mitochondria	0.707	2	0.674–0.740
" histidine	Homogenate	0.422	6	0.367–0.521
	Mitochondria	0.569	5	0.488–0.644
" glutamate, histidine	Homogenate	0.508	7	0.470–0.569
	Mitochondria	1.137	7	1.06 –1.20
Fumarate, glutamate, histidine, PO_4	Homogenate	0.630	9	0.560–0.670
	Mitochondria	1.70	7	1.45 –1.95

in PAH synthesis by mitochondria, while inorganic phosphate produced a further 50 per cent increase.

A study of PAH synthesis in transplantable, C_3H mouse hepatoma 98/15 (14) showed that the amounts of PAH synthesized in the tumor homogenate and mitochondria were too small (0.04 μM of PAH per mg. of N) to warrant further study. Liver homogenate from the animal bearing the transplanted hepatoma synthesized PAH to an extent within the normal range. The addition of normal mouse liver tissue to tumor tissue as a means of supporting oxidative phosphorylation did not result in any significant increase in PAH synthesis over and above that found in the separate tissues. It was therefore concluded that the ability of the liver cell to synthesize PAH was almost completely lost in the transition from the normal to the malignant state.

DISCUSSION

The ideal condition for studying the intracellular distribution of the PAH-synthesizing enzyme system in homogenates would be an environment in which the enzyme system could function at the same maximal rate in all the cell fractions and in the homogenate. The ideal condition is approached experimentally when all the substrate and cofactor requirements have been met and the enzyme system in question can reasonably be regarded as the only variable in the system. In a complex enzyme system, such as the PAH-synthesizing system which we have studied, it is important that no one part of the activity essential for PAH synthesis, such as oxidative phosphorylation, becomes limiting. This would mean that the PAH-synthesizing activities of the nuclear and supernatant fractions which possess relatively little oxidative ability can be properly studied only if supported by an external phosphorylating mechanism which in the distribution studies was provided by the addition of mitochondria.

The substrate requirements of the PAH-synthesizing enzyme system in rat liver, which gave maximal rates of synthesis in the experiments of Cohen and McGilvery, were incomplete for obtaining maximal rates of PAH synthesis in our experiments with mouse liver homogenates and mitochondria in isotonic sucrose. In addition to the need for PAB, glycine, fumarate, K^+, Mg^{++}, ATP, and cytochrome c demonstrated by Cohen and McGilvery, a requirement for an intermediate such as glutamate or α-ketoglutarate, capable of supplying more energy than fumarate alone, and a requirement for inorganic phosphate were also found in the present study. The large activating effect of the boiled supernatant on PAH synthesis by mitochondria in the incomplete system was substantially reduced by the addition of glutamate to the system and the residual activation completely disappeared when inorganic phosphate was added.

That the apparent activating effect of glutamate may be due to the formation of p-aminobenzoylglutamic acid is not borne out by our results. Even if such a compound were formed in appreciable amounts, it was not determined to any significant extent as PAH by the analytical method. A reasonable explanation for the singularly outstanding activating effect of glutamate and α-ketoglutarate may be that phosphorylation coupled with the aerobic oxidation of α-ketoglutarate proceeds at relatively high efficiencies (15–18). We have as yet made no measurements on O_2 consumption to determine whether the over-all P:O ratio during the synthesis of PAH was increased by the addition of glutamate or α-ketoglutarate.

The activation caused by glutamate and inorganic phosphate on PAH synthesis by mitochondria appears to be primarily an effect on the rate of oxidative phosphorylation. In the case of mitochondria, ATP was prob-

ably not a limiting factor in the medium since it appears to act in catalytic amounts (1), and in our experiments the 7' P levels were maintained at relatively high levels even in the incomplete systems. In the mitochondrial systems containing no supernatant inhibitors (M_W or $N_W + M_W$), a direct parallel can be seen between increase in PAH synthesis, on the one hand, and increase in 7' P level, on the other, when glutamate and inorganic phosphate were added to the incomplete systems. These relations indicate that in a dynamic system such as mitochondria, PAH synthesis may be largely determined by the rate of oxidative phosphorylation.

Studies on intracellular distribution of PAH synthesis, together with maintenance of 7' P levels under various conditions, have demonstrated the necessity of establishing conditions for obtaining maximal rates of oxidative phosphorylation in the various cell fractions and their recombinations, as well as in the homogenate in order to obtain maximal rates of PAH synthesis and to obtain valid results on intracellular distribution. With submaximal rates of oxidative phosphorylation, the recovery of PAH-synthesizing activity in the mitochondrial fraction varied from 40 to 75 per cent of the total homogenate synthesis. In the system showing no net loss of 7' P during the incubation, 91 per cent of the total PAH synthesis was accounted for in the mitochondria.

The fact that the major portion of the PAH-synthesizing activity of the mouse liver homogenates was associated with the mitochondria provides further evidence (2–9) for the dominant rôle played by the mitochondria in the metabolism of the liver cell. The present results also clearly demonstrate and again reemphasize that studies of enzymatic mechanisms should employ isolated tissue fractions in the cytologically pure state and that these fractions must be studied in all permutations and combinations in order that the rôle played by each component of the cell can accurately be assessed.

SUMMARY

1. The intracellular distribution of PAH synthesis in C_3H mouse liver homogenates was studied. It was shown that the mitochondria possessed virtually all of the PAH-synthesizing activity of the homogenate.

2. It was demonstrated that in order to obtain maximal rates of PAH synthesis in isolated mitochondria and to a lesser degree in homogenates an oxidizable intermediate such as glutamate or α-ketoglutarate was necessary. A requirement for inorganic phosphate was also shown.

3. A relation between PAH synthesis and rate of oxidative phosphorylation in mitochondria was indicated.

4. PAH synthesis by transplantable, C_3H mouse hepatoma 98/15 was shown to be negligible.

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DEAMINATION OF HOMOSERINE*

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In the study of the enzymatic cleavage of thioethers, it has been found that the heated extracts prepared from liver tissue of rats contain an enzyme active in the deamination of homoserine (1, 2); α-ketobutyric acid has been identified as a product of the action of the extract on homoserine (1). Since it appeared possible that this enzyme was identical with the enzyme (or enzymes) responsible for the deamination of serine and threonine (3, 4), some further study of the enzyme was made.

It was found that highly active preparations of the enzyme did not attack serine or threonine. The enzyme was activated by the addition of adenylic acid and glutathione. There is reason to believe, however, that these materials are not directly concerned with the deamination of homoserine by the enzyme.

EXPERIMENTAL

Methods of Assay—It was found more convenient to determine ammonia released than to follow the formation of the α-ketobutyric acid. Direct nesslerization of a trichloroacetic acid filtrate was found to be satisfactory except in the presence of sulfhydryl compounds such as glutathione; in such cases the aeration described by Archibald (5) was used. DL-Homoserine was incubated with 1 ml. of a solution of the enzyme in a total volume of 10 ml. The buffer was 0.02 M sodium diethyl barbiturate, pH 8.0. At the end of 1 hour of incubation, 0.5 ml. of 50 per cent trichloroacetic acid was added. 5 ml. of the filtrate and 3 ml. of water were placed in a colorimeter tube and 2 ml. of a mixture of equal parts of 1 per cent potassium gluconate and 2.5 per cent potassium persulfate were added. 3 ml. of Nessler's reagent were added and the amount of color was estimated at 470 mμ.

Preparation of Enzyme—Approximately 10 gm. of liver tissue were homogenized with 200 ml. of cold physiological saline, and the mixture, in a 250 ml. centrifuge tube, was heated in a water bath maintained at 58–60°

until the temperature of the mixture reached 55°. The temperature was maintained at 55° for 5 minutes. The mixture was cooled in an ice bath to 4° and the insoluble material was removed by centrifugation. To 1 volume of extract was added 0.5 volume of ethanol (at 4°) and the precipitate was removed and discarded. An additional 0.5 volume of ethanol was added and the precipitate was collected and dissolved in 1 volume of physiological saline. 1 volume of 0.1 M acetate buffer, pH 4.5, was added and the precipitate was removed by centrifugation. The supernatant solution was refractionated with ethanol. The precipitate obtained between 0.5 and 1.0 volumes of ethanol was redissolved in physiological saline containing 0.01 M sodium citrate and 0.001 M NaCN. To the filtered solution was added 0.75 volume of ethanol and the precipitate was discarded. An additional 0.5 volume of ethanol was added and the precipitate was dissolved in saline and filtered. No further purification has

TABLE I

Purification of Homoserine Deaminase

Units are expressed as mM $\times$ 10^{-3} of ammonia released in 1 hour from 4.5 mg. of DL-homoserine under the conditions described in the text.

Step	Total units	Units per mg. protein N
Original extract (unheated)..............................		3
1st alcohol fractionation.................................	2000	51
Acetate precipitation....................................	·1890	910
2nd alcohol fractionation...............................	1340	1070
3rd " "	780	1780

been obtained by fractionation with ethanol. The purification is summarized in Table I.

Specificity of Activity—No activity toward DL-serine or toward DL-threonine was observed under any conditions. No activity was observed with adenylic acid or α-aminobutyric acid as the substrate. The enzymes responsible for the deamination of serine and adenylic acid were removed by the treatment at 50–55°. However, residual thionase activity was found in the most purified preparations. The activity toward homoserine is more rapidly destroyed by heat than is the thionase activity and hence it is possible to use the treatment with heat to remove the "homoserine deaminase" from the thionase (2).

Activation of Enzyme—Several highly purified preparations were found to have little or no activity unless sodium cyanide, sodium citrate, or glutathione was added to the solution of the enzyme. It is believed that this

activation was merely the removal of heavy metal ions from the solution and, in most cases, the activation with these reagents was of a minimal nature. It was found that when the distilled water used in the preparation
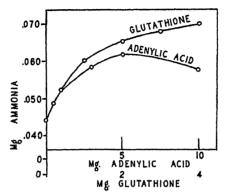
FIG. 1. Activation of the deamination of homoserine by adenylic acid and glutathione. 0.08 mM of DL-homoserine was incubated as described in the text.

TABLE II

Optical Specificity of Enzymatic Activity

The substrates were incubated with 1 ml. of enzyme preparation (0.0006 mg. of protein N) in a total volume of 10 ml. for 60 minutes. The buffer was 0.02 M sodium diethyl barbiturate, pH 8.0. The lactones were neutralized by the addition of the calculated amount of sodium bicarbonate.

Substrate	Concentration	Ammonia released
	m_M	m_M
DL-Homoserine............................	0.08	0.0048
L-Homoserine.............................	0.04	0.0067
D-Homoserine.............................	0.04	0.0001
L-Homoserine.............................	0.04	0.0032
+ D-Homoserine..........................	0.08	
DL-Homoserine lactone HBr...............	0.08	0.0002
" " HCl...............	0.08	0.0002
L-Homoserine " HBr...............	0.04	0.0001
D-Homoserine " "	0.04	0.0001

of solutions was passed through ion exchange resins[1] the effects of these agents were minimized.

[1] Deeminizer, Crystalab, Crystal Research Laboratories, Inc., Hartford, Connecticut.

The effects of the addition of adenylic acid and glutathione to a preparation are illustrated in Fig. 1. It is apparent that, at this pH, adenylic acid is an activator.

Optical Specificity—D-Homoserine was not attacked by the enzyme and, in addition, was found to be an inhibitor of the action on L-homoserine. These studies are summarized in Table II. It is of interest that if adequate precautions were taken to prevent the opening of the lactones no activity toward DL-, L-, or D-homoserine lactone was observed. Considerable time was expended in an effort to find an enzyme which might catalyze the conversion of the lactone to homoserine but no indication of such activity was obtained.
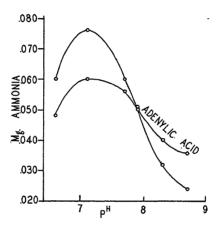
FIG. 2. Effect of pH on the deamination of homoserine. 0.08 mM of DL-homoserine was incubated in 0.02 M phosphate buffers for 1 hour.

pH-Activity Relationships—All the above studies were made with a sodium diethyl barbiturate solution as the buffer in order to rule out possible effects of inorganic phosphate. Since such buffers could not be used in the study of the effect of pH on the activity of the enzyme, phosphate was selected. In addition to the study of the enzyme in the presence of glutathione and adenylic acid, a study was made in the absence of adenylic acid. The results are illustrated in Fig. 2. It is obvious that adenylic acid inhibited the enzyme except at higher values of pH. It is doubtful, therefore, whether adenylic acid has any function in the deamination of homoserine.

SUMMARY

An enzyme of liver tissue of rats, responsible for the deamination of homoserine, has been obtained in a state of considerable purification. The

enzyme does not attack serine or threonine. Some activation with gluta-thione and adenylic acid was observed, but neither substance appears to be essential for the deamination of homoserine. The enzyme did not attack D-homoserine or the lactones of L-homoserine or DL-homoserine.

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A STUDY OF XANTHINE METABOLISM IN THE RAT*

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For many years it has been known that purine catabolism in the rat results in the formation and subsequent urinary excretion of allantoin as the main end-product. Individual enzymes catalyzing the steps in purine catabolism have been identified and methods for assaying these enzymes *in vitro* have been developed. With the discovery that xanthine oxidase activity *in vitro* is influenced greatly by the level of dietary protein (1–3) a means was offered for directly comparing the activity *in vitro* of the enzymes involved in xanthine catabolism with activity of those enzymes in the intact animal.

In rats on low protein diets it has been observed that liver xanthine oxidase activity as measured *in vitro* is greatly decreased, in many cases disappearing entirely, if the dietary protein level is reduced sufficiently. If xanthine oxidase rather than uricase limited the rate of breakdown of xanthine to allantoin, animals with low xanthine oxidase activity should convert xanthine to allantoin at a slower rate than animals with normal xanthine oxidase activity. If not, then measurements *in vitro* of xanthine oxidase activity may not be taken as an indication of the actual rate of xanthine oxidation in the intact animal.

This problem was studied by placing animals on a ration known to decrease xanthine oxidase activity *in vitro*, by injecting them with a known amount of xanthine, measuring the rate of allantoin excretion in the urine, and finally assaying the tissues of the rats for xanthine oxidase and uricase activity. The urinary excretion of other purines from the animals with xanthine injected was also measured in an attempt to account for all of the injected xanthine either as allantoin or purine. The xanthine was injected rather than fed, since dietary xanthine would probably be incompletely absorbed from the intestinal tract because of its relatively low solubility. In that case quantitative relationships between the amount of xanthine ingested and catabolites excreted could not have been made.

EXPERIMENTAL

Two groups of male rats of the Holtzman strain, weighing 60 to 70 gm., were given synthetic rations for 3 weeks before use in the enzyme studies. One group of ten animals received a 6 per cent casein ration and the other group of nine animals an 18 per cent casein ration plus 0.25 per cent DL-methionine. The common components of the two rations were as follows: vitamin mixture previously described (1) 2 per cent, Salts IV (4) 4 per cent, corn oil 5 per cent, respective casein level, and sucrose to make 100 per cent. In addition, 2 drops of fortified haliver oil per week were given orally to each animal. At the end of the 3 week feeding period one animal from each group was sacrificed for determinations of liver xanthine oxidase to make certain that the expected drop in liver activity in the rats on the low protein ration had occurred. The remaining animals of both groups were then placed in metabolism cages, two animals per cage, for urine collections. Basal urine excretions were collected under toluene for a 93 hour period. At the end of this time each animal was injected intraperitoneally with a 3 ml. suspension of 25 mg. of xanthine per 100 gm. of body weight. The xanthine was dissolved first in equivalent sodium hydroxide and then brought to pH 7.3 in order to obtain a finely divided suspension. The rats were replaced in the metabolism cages and fed their respective synthetic rations and given water *ad libitum*. Urine collections were made thereafter at the following intervals from the time of injection: 3, 6.5, 11, 22, 27, 31, 46.5, 53, 70.5, 77, 94.5, and 143 hours.

The urine samples were neutralized to pH 7.0, evaporated to dryness, extracted with small portions of hot water, filtered, and made to 10 ml. Allantoin was determined in each sample by the method of Young and Conway (5). Because of the somewhat unstable nature of allantoin, recoveries of allantoin added to the urines were made in several cases. These averaged 103 ± 0.1 per cent recovery.[1] Xanthine and guanine were determined in the urines by the method recently developed by one of the authors (6). Uric acid was determined in the urines by a modification of Brown's method (7). Adenine was not determined because of lack of an adequate micromethod for adenine and because adenase has not been observed in rat tissues, making it doubtful that the injected xanthine would be converted to adenine.

After the final urine collections were made, the rats were sacrificed and xanthine oxidase activity of the liver determined according to the method of Axelrod and Elvehjem (8). It also appeared necessary to measure the xanthine oxidase activity of the kidney as well as of the liver, since considerable xanthine oxidation is believed to occur in kidney as well as in

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[1] Standard error of the mean.

liver. The manometric method used for measuring liver xanthine oxidase activity, however, could not be employed for measuring kidney activity (8). Therefore, a new method was devised by which kidney xanthine oxidase activity was readily measured.

The following procedure was adopted for assaying kidney xanthine oxidase: 2 ml. of a 1:6 kidney homogenate in 0.1 M sodium phosphate buffer (pH 7.3) were mixed with 4 ml. of a salicylaldehyde solution (1 mg. per ml.) and 2 ml. of the phosphate buffer in a 125 ml. Erlenmeyer flask. A blank was also prepared containing 4 ml. of water in place of the salicylaldehyde. 1 ml. of the mixture was withdrawn immediately and pipetted into a solution of 2 ml. of water, 1 ml. of 0.6 N sulfuric acid, and 1 ml. of 10 per cent sodium tungstate. The rest of the enzyme mixture was lightly stoppered and incubated at 37° for 90 minutes. At the end of that time a 1 ml. aliquot was withdrawn and treated similarly to the pre-incubation aliquot.

The tungstate precipitates were centrifuged and the supernatant was filtered. 2 ml. aliquots of the filtrate were treated with 0.1 ml. of Folin-Ciocalteu phenol reagent (9) and 1 ml. of 1 N sodium hydroxide. Color was allowed to develop for 5 minutes, 6 ml. of water were added, and the tubes were read in an Evelyn colorimeter with a 620 $m\mu$ filter. A series of salicylic acid standards (0 to 100 γ) was treated in a similar manner. Under the conditions employed salicylic acid gives a deep blue color, while salicylaldehyde gives only an insignificant response.

It was considered necessary to observe the effects of a low protein ration upon uricase activity *in vitro* as well as xanthine oxidase activity, since both of these enzymes are involved in the oxidation of xanthine to allantoin. Therefore, two groups of rats of four animals each were given the 6 per cent casein and 18 per cent casein + 0.25 per cent DL-methionine rations, respectively, for the same length of time as the animals previously used in the xanthine oxidase studies. Uricase activity was determined in the liver of each animal with lithium urate as substrate (8). Kidney uricase activity could not be adequately determined by this method.

RESULTS AND DISCUSSION

The results for the enzyme activities as measured *in vitro* are presented in Table I. Enzyme activities of both xanthine oxidase and uricase of liver are expressed as microliters of oxygen uptake at 30° due to added substrate per hour per gm. of wet liver. Kidney xanthine oxidase activity is expressed as microliters of oxygen uptake equivalent to the amount of salicylaldehyde oxidized to salicylic acid per hour per gm. of wet tissue at 37°.

From these results it may be seen that a diet containing 6 per cent pro-

tein is not adequate to maintain either liver or kidney xanthine oxidase activity at a normal level.   In fact, liver xanthine oxidase activity is decreased almost to zero, while kidney xanthine oxidase activity is decreased to less than half that found in animals fed an 18 per cent casein ration supplemented with methionine.   These results verify previous work reported by two of the authors (1) in which it was suggested that liver xanthine oxidase activity is a sensitive index of protein balance.   The method used for the estimation *in vitro* of liver xanthine oxidase has been checked against other methods for assaying that enzyme by Richert *et al.* (10) and the results were in good agreement with those obtained by other methods reported in the literature.   Therefore, it is believed that the decrease in liver xanthine oxidase observed in these experiments is not a function of the method employed but a true function of the levels of dietary protein.

TABLE I

Effect of Level of Dietary Protein upon Liver and Kidney Xanthine Oxidase and Uricase Activity in Rats

Ration	No. of animals	Enzyme determined	Activity*	
			Liver	Kidney
6% casein	9	Xanthine oxidase	1.30 (0– 13)†	37.2 (25.6–44.8)
	4	Uricase	390 (246–530)	
18% casein + 0.25% DL-methionine	8	Xanthine oxidase	110 (80–143)	82.0 (59.0–98.4)
	4	Uricase	600 (420–780)	

* See the text for units of enzyme activity.
† Range.

The salicylaldehyde method for kidney xanthine oxidase outlined in the foregoing was checked with the livers of the same animals in which the values for kidney activity are reported.   This method, when employed for liver, was found to correlate very well with the manometric method.

From Table I liver uricase activity was observed to be decreased by the low protein diet, although not as much as xanthine oxidase.   The fact that the actual activity of uricase in either group of animals is much greater than xanthine oxidase activity in oxygen uptake per gm. of liver leads one to believe that, at least *in vitro*, uricase is not the limiting step in the catabolism of xanthine.   Xanthine oxidase appears to be much more limiting than uricase.   This same conclusion was drawn by Axelrod and Elvehjem in other deficiency studies in the rat (8).

In Fig. 1 are reported the values obtained for excretion of allantoin, uric acid, xanthine, and guanine after the single intraperitoneal injection of

xanthine. To obtain the curves presented in Fig. 1 the excretion values for all the animals of each group were averaged and plotted against the time of the urine collections, each value being added to the previous value to give curves analogous to "oxygen uptake curves." In every case the basal excretion per hour, obtained prior to the xanthine injected, was multiplied by the time in hours of each collection and subtracted from the
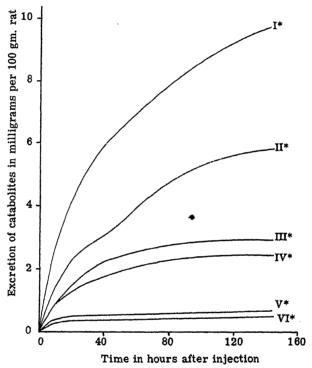
FIG. 1. The rates of excretion of various purine catabolites after injection of xanthine. Curves I*, V*, and VI*; excretion of allantoin, uric acid, and xanthine plus guanine, respectively, by rats fed the 6 per cent casein ration; Curves II*, III*, and IV*, excretion of allantoin, xanthine plus guanine, and uric acid, respectively, by rats fed the 18 per cent casein + 0.25 per cent methionine ration.

total excretion obtained over that time interval. Consequently, the excretions depicted in Fig. 1 are due only to metabolism of the injected xanthine. The ordinates of the curves represent excretion of the various catabolites in mg. per 100 gm. of body weight of rat. It was necessary to make all animals to the same weight by this means, since at the time of injection the rats receiving the higher protein level were considerably

heavier than those receiving the 6 per cent level. Even if this had not been done, the curves would still lie in the same order with respect to each other.

It can be seen from the allantoin excretion curves, that the animals with low liver and kidney xanthine oxidase as measured *in vitro* are able to convert injected xanthine to allantoin at a rate nearly double that of the animals with normal xanthine oxidase activity *in vitro*. Moreover, from Table I it may be observed that uricase activity is not limiting in either group of animals when compared to xanthine oxidase activity. Therefore, it appears that activity of the enzymes investigated in this study when measured *in vitro* may not be a true indication of the actual rate of xanthine oxidation in the intact animal.

From the excretion curves of uric acid from the two groups of animals, it appears that uric acid is spilled over more easily into the urine of the rats on the higher protein ration than of those fed the 6 per cent protein ration; this is also true in the case of xanthine and guanine. The signifi-

TABLE II

Urinary Excretion of Allantoin and Purines after Injection of Xanthine

Ration	Xanthine injected	Allantoin excreted	Uric acid excreted	Xanthine + guanine excreted	Total catabolites excreted
	μM	μM	μM	μM	μM
6% casein..........................	164	69.5	5.95	5.60	81.1
18% casein + 0.25% DL-methionine....	164	35.4	13.4	19.5	68.3

cance of these findings is not clear unless the level of dietary protein produces a change in the renal thresholds of these substances. The uricase estimations *in vitro* do not appear to be directly involved in the results obtained for the excretion of uric acid. The rats receiving the higher protein level excrete more uric acid even though their liver uricase activity is also higher than that of the other group of rats. These results appear to be related to the well known observation that the liver of the Dalmatian dog is fairly rich in uricase, although it excretes most of its uric acid unchanged (11).

At the end of 143 hours the excretion curves have leveled off almost completely, except for allantoin from the animals fed 6 per cent casein. This curve, however, if continued with the same curvature, becomes asymptotic with a level of about 11 mg. of allantoin. If one adds up the total excretion of allantoin and the purines measured, the results shown in Table II are obtained. In Table II the values are expressed in micromoles in order to place all of the substances on an equivalent basis. It thus ap-

pears that only 49 per cent of the injected xanthine can be accounted for by the total excretion from the rats given 6 per cent protein and only 41 per cent from the group receiving 18 per cent protein. These somewhat unusual results cannot be explained by possible destruction of the catabolites by the treatment given the urines, since the only fairly unstable one, allantoin, was found to give excellent recovery values when added directly to samples of the urines (see "Experimental"). One possible explanation for our results is that the xanthine entered some metabolic pathway other than those it is known to enter. For example, the relatively large concentration of xanthine given the animals in the single injection may have reversed by mass action the reactions by which purines are synthesized. Another possible explanation is that some of the injected xanthine was incorporated via guanine into the nucleic acids of the animal. However, Brown, Roll, and Plentl (12) have shown in this respect that dietary guanine is not incorporated into body nucleic acids, although dietary adenine may be. This point remains to be elucidated. Probably the best attack would be through the use of isotopically labeled xanthine.

SUMMARY

Results have been presented which demonstrate that activity of an enzyme (xanthine oxidase) measured *in vitro* is not a true indication of rate of xanthine catabolism in the intact animal. Moreover, less than half of the xanthine injected intraperitoneally in a single large dose can be accounted for as conventionally expected catabolites.

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THE INFLUENCE OF FOLIC ACID UPON ENZYME SYSTEMS IN CHICK EMBRYO LIVER*

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The influence of folic acid upon various enzyme systems both *in vivo* and *in vitro* has recently been investigated by workers in several laboratories. In this laboratory it has been demonstrated that folic acid depresses xanthine oxidase activity when added to the enzyme systems *in vitro* (1) or when fed to chicks (2). It has also been shown that D-amino acid oxidase activity is decreased by dietary folic acid in the chick (2) and in the rat (3). Whereas endogenous respiration of liver is strongly inhibited by folic acid added to a liver homogenate *in vitro* (1), this effect was not observed when folic acid was fed to chicks (2). Dinning, Keith, and Day (4) have recently observed that aminopterin, an antimetabolite of folic acid, when fed to monkeys, causes a marked decrease in choline oxidase activity of the liver and kidney. Similar results were obtained in the monkey by a dietary restriction of folic acid. They suggest that folic acid functions as a constituent of the prosthetic group in the choline oxidase enzyme system.

Investigating the effect of folic acid upon the hatchability of eggs, we have attempted to elucidate the mechanism by which hatchability is increased either by feeding folic acid to the hen or by injecting the vitamin into eggs produced by folic acid-deficient hens. The effect of folic acid on hatchability has previously been reported by Sunde *et al.* (5). In the present investigation the effects of folic acid on several liver enzyme systems of chick embryos have been studied. Because folic acid appears to function in purine metabolism, the effect of the vitamin on xanthine oxidase activity and uric acid content of embryo liver was studied. In order to observe the effects of the vitamin on what is believed to be a measure of general metabolic rate of the liver, endogenous respiration of the livers were determined. Since it has been observed that the ratio of liver weight to body weight in the chick is decreased by dietary folic acid (2, 6),

and since the same effect was found to occur with chick embryos in this investigation, liver nitrogen per gm. of fresh liver was determined. Thus if the relatively large size of the liver in relation to body weight is caused by dilution of the liver with water, fat, or other substances, this fact will be demonstrated by liver nitrogen determinations. On the other hand, if liver nitrogen is retained in preference to body stores even in a folic acid deficiency, demonstration of this effect would be of fundamental importance. In relation to the work of Dinning et al. (4) it was decided to measure choline oxidase activity of the chick embryo livers as a function of the effect of folic acid.

<div align="center">EXPERIMENTAL</div>

The management of the hens has been described previously by Cravens and Halpin (7). Two groups of four single comb white Leghorn pullets were placed on rations containing 3000 γ and 150 γ of folic acid per kilo of ration, respectively. The basal ration consisted, in per cent, of hot alcohol-extracted casein 18, gelatin 5, Salts IV (8) 5, soy bean oil 3, vitamin A and D feeding oil (400 units of vitamin D, 3000 units of vitamin A) 2, fish solubles 3, and sucrose 64. Crystalline vitamins were added as follows in mg. per kilo of basal ration: thiamine hydrochloride 4, riboflavin 6, niacin 100, pyridoxine hydrochloride 4, calcium pantothenate 15, choline chloride 2000, biotin 0.2, inositol 1000, α-tocopherol 3, and menadione 0.5.

The eggs from the two groups of hens were collected and incubated conventionally. When groups of eggs were to be injected with folic acid, a small hole was drilled in the large end of the egg and the vitamin was injected into the albumin adjacent to the yolk. In all cases the volume of the vitamin solution injected was 0.1 ml. containing 25 γ of folic acid.

After the eggs were opened, the livers were removed from the embryos, chilled in cracked ice for several minutes, blotted on filter paper, and weighed on a sensitive torsion balance. Since in most cases the livers were too small to be used singly for the determinations, two and sometimes three livers were pooled from embryos of the same group. For the xanthine oxidase determinations the livers were added to 5 volumes of 0.039 M sodium-potassium phosphate buffer (pH 7.3), homogenized for 1 minute, and strained through gauze. The xanthine oxidase activity was then determined according to a method previously outlined by Keith et al. (6). Uric acid was determined in an aliquot of each homogenate by a modification of the procedure of Brown (9).

For the choline oxidase determinations the embryo livers were homogenized in 2 volumes of the phosphate buffer. 0.5 ml. portions of the resulting 33 per cent homogenate were incubated for 10 minutes with the conventional Warburg apparatus. Then 0.5 ml. of choline chloride solu-

tion (5 mg. per ml.) or 0.5 ml. of water for the control flask was added from the side arm, the stop-cocks were closed, and a reading of the control flasks was taken at the end of 10 minutes. The 10 minute reading was used as a measure of endogenous liver respiration. Readings of all the flasks were then taken at 20 minute intervals. When the oxygen uptake due to oxidation of choline was plotted against time, linearity for 60 minutes was obtained in almost every case. Therefore, choline oxidase activity was calculated from the total oxygen uptake due to the oxidation of choline after 60 minutes.

Aliquots of each homogenate were taken for nitrogen analysis by a semi-micro-Kjeldahl procedure. Activity of the enzymes determined was calculated both in terms of liver fresh weight and liver nitrogen. The amount

TABLE I

Effect of Dietary Folic Acid in Hen upon Enzyme Systems in Liver of Embryos

Enzyme determined	No. of embryos	Folic acid ration of hens	Activity of embryo liver based on	
			Liver fresh weight	Liver N
			γ uric acid formed per hr. per gm. liver	*γ uric acid formed per hr. per mg. liver N*
Xanthine oxidase	13	Normal	208 ± 33*	9.1 ± 1.4
	24	Low	510 ± 70	22.2 ± 3
			μl. O₂ per hr. per gm. liver	*μl. O₂ per hr. per mg. liver N*
Endogenous respiration	9	Normal	1750 ± 170	82 ± 8
	19	Low	1780 ± 30	83 ± 1
Choline oxidase	9	Normal	330 ± 16	15.5 ± 0.7
	19	Low	510 ± 14	24.0 ± 0.6

* Standard error of the mean.

of nitrogen per gm. of liver was obtained from the homogenate nitrogen determinations.

RESULTS AND DISCUSSION

In Table I are presented the results of the enzyme determinations from the livers of embryos from the hens receiving 3000 and 150 γ of folic acid per kilo of ration, respectively. The typical depression of xanthine oxidase activity by dietary folic acid earlier observed in chicks (2) was observed in the embryos from hens fed normal levels of folic acid. Moreover the decrease in xanthine oxidase activity in the embryo liver is just as pronounced as that observed in the livers of chicks fed folic acid (2). Therefore, if the effect of folic acid *in vivo* acts to inhibit directly the enzyme system as it does *in vitro*, enough of the vitamin would have to be transferred from the hen to the egg, then to the embryo, to inhibit the enzyme activity to the same extent as feeding folic acid directly to chicks.

However, it can be observed from Table I that folic acid fed to the hen did not inhibit the endogenous respiration of the embryo liver, although if folic acid is added *in vitro* to liver, endogenous respiration is markedly decreased. Therefore, it appears that the folic acid transferred to the egg is transferred in a form still capable of depressing xanthine oxidase but incapable of inhibiting endogenous respiration. Another possibility is that the folic acid transferred to the embryo from the egg acts as a regulator of enzyme protein formation and that, in the case of xanthine oxidase, the presence of folic acid decreases the amount of xanthine oxidase protein synthesized by the embryo. We have evidence (to be published shortly) that such an explanation holds true for the D-amino acid oxidase system of liver. It has been noted that dietary folic acid markedly depresses liver D-amino acid oxidase activity (2). Our new evidence indicates that, when folic acid is added to a liver homogenate *in vitro*, it has no effect on

TABLE II

Effect of Folic Acid Injected into Eggs from Folic Acid-Deficient Hens upon Choline Oxidase Activity of Embryo Livers

Day of incubation eggs were injected	No. of embryos	Folic acid injected	Choline oxidase activity	
			Based on fresh liver weight	Based on liver N
			μl. O₂ per hr. per gm. liver	*μl. O₂ per hr. per mg. liver N*
0	7	+	575 ± 66	25 ± 2.7
	8	−	690 ± 39	30 ± 1.5
15	12	+	570 ± 27	21.8 ± 1.0
	11	−	560 ± 49	22.6 ± 1.9

the D-amino acid oxidase activity of the liver. Thus it appears that dietary folic acid, while in some cases bringing about the same effect on enzyme systems, follows a mechanism of action different from folic acid added to enzyme system *in vitro*.

From Table I embryo liver choline oxidase appears to be depressed by folic acid fed to the mother hen. The extent of depression is almost the same as that noted in the case of xanthine oxidase activity. Dinning *et al.* (4) were unable to obtain any consistent effect upon choline oxidase activity by folic acid *in vitro*. Hence it appears that, while chick embryo choline oxidase may be inhibited by folic acid fed to the hen, folic acid has a different effect on the activity of that enzyme when added *in vitro*. This conclusion is similar to that mentioned in the preceding section concerning the effects of folic acid *in vitro* and *in vivo* on endogenous respiration and D-amino acid oxidase activity.

In Table II are presented the results demonstrating the effect of folic

acid on choline oxidase activity when the vitamin was injected into eggs from folic acid-deficient hens. When folic acid was injected at 0 day of incubation and the eggs were incubated until the 19th day, a slightly depressant effect of folic acid on liver choline oxidase activity was observed. However, if the folic acid was injected on the 15th day after laying and liver choline oxidase activity determined on the 19th day, the folic acid appeared to have no effect on activity of the enzyme. The apparent difference in the absolute choline oxidase activities of the two groups not injected with folic acid (Table II) should not be taken as an actual discrepancy in the enzyme results since those two groups of eggs were obtained at widely different time intervals from the hens. However, the eggs from the groups incubated for the same length of time were obtained at the same time and the choline oxidase activities run on a single day. Therefore, from the results of Tables I and II concerning choline oxidase activity, it appears that at least 19 days are necessary for the full effect of folic acid on choline oxidase activity to become evident. The greatest effect appears if the folic acid is fed to the hen and then transferred to the egg, a slight effect occurs if the folic acid is injected prior to incubation, and no effect appears if only 4 days are allowed for the effect of the folic acid to occur. A possible explanation for these results is that one rôle of the folic acid in the embryo is to regulate synthesis of the enzyme protein rather than to have a direct inhibitory effect on the enzyme. Otherwise the greatest inhibitory effect should have been observed in the embryos injected with the vitamin on the 15th day of incubation, which would more nearly parallel an effect *in vitro*. In this connection it might be mentioned that Snell and Cravens (10) have recently observed that the toxicity of aminopterin to the developing chick embryo which can be partially reversed by the desoxyribosides of thymine or hypoxanthine is most effective when injected into the eggs prior to incubation. The effectiveness of the toxicity decreases as embryonic development progresses. Similarly, Sunde *et al.* (11) have recently shown that the earlier the eggs from folic acid-deficient hens are injected with folic acid, the longer the chicks that hatch will live. Therefore, it appears that with respect to choline oxidase activity, desoxyriboside synthesis, and chick viability, folic acid appears to have its most pronounced effects early in the development of the embryo.

The effects of folic acid on liver nitrogen and uric acid content are given in Table III. It appears that the vitamin has little obvious effect upon synthesis of uric acid, though xanthine oxidase activity is affected by folic acid. Similarly the amount of nitrogen per gm. of liver is unchanged, whether folic acid is fed to the mother hen or not. This negative effect upon liver nitrogen content is important, however, since in relation to total body weight the livers of folic acid-deficient chicks and embryos are

arger than those given folic acid.   Therefore, the total amount of nitrogen
n the livers of the embryos from folic acid-deficient hens is relatively
greater than that of the embryos from hens fed normal levels of the vita-
nin.   This indicates that in a folic acid deficiency nitrogen apparently is
ncorporated into the liver regardless of the needs of the rest of the body.
[n relation to protein metabolism this possibly means that folic acid plays
an important rôle as a regulator of protein synthesis in the organism and

TABLE III

Influence of Folic Acid upon Liver Nitrogen and Uric Acid in Chick Embryos

Component measured	No. of embryos	Folic acid ration of hens	Amount per gm. wet liver
			gm. N per gm. liver
Nitrogen................	17	Normal	0.0224
	43	Low	0.0225
			mg. uric acid per gm. liver
Uric acid................	13	Normal	0.218
	24	Low	0.220

that these effects are demonstrated by the differences observed in activity
of the various enzymes reported in this paper.

SUMMARY
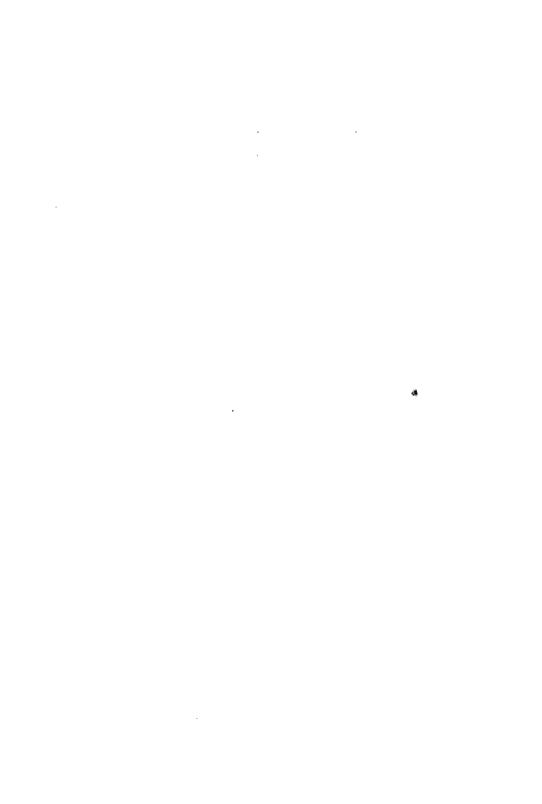
The effects of folic acid upon xanthine oxidase, endogenous respiration,
and choline oxidase activity of embryo liver from incubated eggs produced
by hens either deficient in or given dietary folic acid have been reported.
In addition the influence of folic acid upon embryo liver nitrogen and uric
acid has been presented.   Implications of the function of folic acid as a
possible regulator of protein metabolism have been discussed.   It appears
from our data and also those of others that some of the most pronounced
effects of folic acid occur early in the development of the embryo.

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AMYLASE AND ESTERASE IN RAT INTESTINAL LYMPH

BY EUNICE V. FLOCK AND JESSE L. BOLLMAN

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Alkaline phosphatase, which occurs in very high concentration in the intestinal mucosa (1) and is excreted (2) in the intestine in large quantities during digestion, has been found also in increased concentration in the plasma (3) and especially in the intestinal lymph (4) of the rat after it has been fed a meal containing fat. This increase in the alkaline phosphatase activity of intestinal lymph is greatly diminished or abolished if the bile is excluded from the intestine (5). To determine whether other enzymes involved in the digestion of food are transported in increased amount in the intestinal lymph after feeding, we have made a study of the amylase and lipase activity of this lymph before and after feeding. The fluctuations found in the concentration of these enzymes at various times do not appear to be correlated with the time of feeding. A definite increase in the aliesterase which hydrolyzes tributyrin was found after the feeding of a meal containing fat.

Methods

Male rats of the Sprague-Dawley strain, weighing approximately 200 gm. and maintained on Friskies, were used in these studies. They were fasted 20 hours. Then, with the animals under ether anesthesia, polyethylene tubing was inserted into an intestinal lymphatic trunk in the mesentery just prior to its entrance into the cisterna chyli as previously described (6). The rats were placed in small cages (7) with free access to water which contained 0.2 per cent sodium chloride to compensate partially for the loss of salt in the lymph, which was collected continuously. On the day after operation the rats were given either a fat-free or a fat-containing (4) meal by stomach tube. Lymph was collected in periods of 2 and 4 hours each and then, to assure a prolonged period of digestion and absorption, a second meal was given and lymph was collected for an additional period of 18 hours. Blood was removed at the end of the experiment by cardiac puncture.

Because of the very high concentration of fat often found in lymph, the amylase can best be determined by measurement of the sugar liberated by hydrolysis of starch. The amylase activity of lymph or plasma which had been diluted 10- to 25-fold was measured by the ferricyanide method

of Norby as modified by Lagerlöf (8), without preliminary removal of proteins. 1 mg. of maltose liberated from soluble starch in 15 minutes at 37° represents a unit of amylase activity. The esterase activity on the substrate tributyrin was measured by the method of Goldstein, Epstein, and Roe (9). The liberated fatty acids were titrated with 0.05 N potassium hydroxide to pH 10.65 as measured with the glass electrode. A unit of esterase activity refers to 1 ml. of 0.05 N potassium hydroxide used in this titration. In some experiments olive oil was used as a second substrate, emulsified with desoxycholic acid according to the method of Maclay (10) or with a solution of acacia according to that of Nothman, Pratt, and Benotti (11).

The total secretion of enzyme is obtained by multiplying the volume of lymph by the concentration of enzyme as measured by its activity. The output during the fasting period from the time of operation to the first meal was calculated to a 24 hour period when necessary for comparison.

Results

The mean amylase activity of forty-eight samples of intestinal lymph of fourteen rats collected during the fasting period and the three periods after the feeding of either the fat-containing or the fat-free meals was 19.5 units per ml. with a standard deviation of 9.3. The variations in amylase among the rats at any given period or in a single rat at the different periods were similar and were not related either to the type of meal fed or to the time after feeding.

The total amount of amylase in a 24 hour collection of intestinal lymph was greater when the rats were fed than when they were fasted. Increases of 145 and 48 per cent were found after administration of the fat-containing and fat-free meals respectively (Table I). The increased secretion of amylase was in large measure merely a reflection of the increased volume of lymph, which was 85 and 28 per cent greater after each of the two kinds of meals.

The mean tributyrinase activity of the intestinal lymph of twenty-nine fasting rats was 2.37 units per ml. with a standard deviation of 1.15. There was no appreciable change in the concentration of this enzyme after administration of the fat-free meals, but there was a definite increase after administration of the fat-containing meals. A large increase in the total amount of tributyrinase in a 24 hour collection of lymph was found after the administration of the fat-containing meals, as compared with the fasting period (Table I). The mean increase of 364 per cent in the output of this enzyme was greater than the 96 per cent increase in the lymph volume. The increase in output of tributyrinase after administration of the fat-free meals was similar to that in the lymph volume.

The amount of fatty acids liberated from olive oil during either a 3 (11)

or an 18 hour (10) period of incubation with lymph was generally smaller than the amount liberated from tributyrin in a 1 hour period of incubation, and the amount did not vary appreciably with feeding a fat meal. The

TABLE I

24 Hour Volume and Enzyme Content of Intestinal Lymph

No. of rats	Fat-containing (+) or fat-free (−) meals	Lymph volume, ml.		Enzyme content, units	
		Fasting	Fed	Fasting	Fed
				Amylase	
6	+	16.4 ± 2.6*	30.4 ± 3.6	258 ± 37	632 ± 104
17	−	15.0 ± 1.7 (14)†	19.2 ± 2.0	230 ± 26	340 ± 33 (15)
				Tributyrinase	
20	+	14.7 ± 1.6	28.8 ± 1.9	27.8 ± 2.7	129 ± 13
19	−	14.5 ± 1.5 (17)	16.7 ± 1.4	26.6 ± 2.9 (18)	32.2 ± 3.3
				Lipase (olive oil substrate)	
10	+	13.7 ± 2.6	36.7 ± 2.5	19.2 ± 3.8	33.4 ± 2.5

* Mean ± standard error.

† The numbers in parentheses represent the number of rats when this differs from the number in the left column.

TABLE II

Tributyrinase Content of Plasma

No. of Rats	Hrs. after meal	Tributyrinase, units per ml.
	Normal rats	
10	24	5.36 ± 0.29*
31	0, 2, 4, 6	5.10 ± 0.22
4	24 (18)†	5.06 ± 0.34
	Rats losing intestinal lymph for 48 hrs.	
31	24 (18)†	2.67 ± 0.15

* Mean ± standard error.

† 24 hours after first meal, 18 hours after second meal.

total secretion of this lipase during the 24 hour feeding period did not increase as much as did the lymph volume (Table I).

The mean tributyrinase activity of the plasma of normal intact rats varied from 5.06 to 5.36 units per ml. whether the rats were fed or fasted

(Table II). Tuba and Hoare (12) have found no appreciable change in the activity of this enzyme in the serum of rats after a 5 day fast. The plasma levels that we found were more than twice as great as those of the intestinal lymph of the fasting rat, but smaller than those of lymph after administration of the fat meals. After external drainage of the intestinal lymph for periods of 48 hours, the activity of this enzyme in the plasma was reduced to 2.67 units.

The amylase activity of the plasma was 42.9 ± 3.2 (standard error) units per ml. in normal intact rats fed Friskies *ad libitum* and 24.1 ± 1.5 units after a 24 hour fast. When normal intact rats were fasted for 1 day and then fed two fat-free or fat-containing meals by stomach tube in 1 day, the amylase activity of the plasma averaged 30.1 ± 1.1 units at 2, 4, 6, or 24 hours after the first meal. The plasma level was not affected significantly by the external drainage of intestinal lymph for 48 hours. At this time the level in the plasma was 28.0 ± 2.0 units compared to 21.8 ± 3.3 units in the lymph.

Comment

The concentration of amylase circulating in the plasma of the rat is very high (13), being 2 to 4 times as great as that in the dog, which in turn is 5 to 20 times that of the human subject (14). Concentrations of amylase in the intestinal lymph of the rat are generally lower than those in the plasma, as had been found for the thoracic duct lymph of the dog by Carlson and Luckhardt (15). Fluctuations occurred in the concentration of amylase in the intestinal lymph throughout the fasting and feeding periods studied but were not related to the time of feeding. This indicates that amylase is not absorbed from the intestine into the intestinal lymph during the digestion of food, nor is it secreted in increased amounts into the lymphatic vessels of the pancreas when this gland is secreting enzymes into the intestine, since pancreatic lymph is included in our intestinal lymph and any appreciable influx of enzyme from this source should be detectable. The total amount of amylase secreted in the intestinal lymph varies with the volume of lymph secreted and thus reflects the ability of the organism to maintain a high degree of uniformity in the chemical composition of intestinal lymph even when the rate of formation of the lymph is greatly changed by variations in food and water intake. The amylase of the lymph probably represents that which has diffused into the lymph spaces from the blood. The concentration of amylase in the plasma is maintained very well even when intestinal lymph has been drained externally for a 2 day period.

The concentration of tributyrinase in the intestinal lymph of the fasting rat is much smaller than that of the plasma. It is not changed after the feeding of a fat-free meal but does increase markedly after the feeding of

a fat-containing meal. The enzymatic activity of fasting lymph toward tributyrin may be due in part to pancreatic lipase or S-cholinesterase as well as aliesterase, since all these enzymes are able to hydrolyze tributyrin, but the increased activity after administration of a fat meal appears to be due to the aliesterase. Pancreatic lipase can be ruled out as the source of the increased enzymatic activity of lymph after administration of fat-containing meals, since no increase in the activity of lymph toward olive oil was found. Cholinesterase can also be excluded, since Zeller and Mc-Naughton[1] have found no increase in the hydrolysis of acetylcholine by the lymph of these rats after fat feeding. Hess and Viollier (16) have found that the tributyrinase content of blood plasma was markedly decreased in rats maintained for 3 weeks on a fat-free diet, when there was no change in the cholinesterase content.

The increase in tributyrinase secreted after the feeding of a fat-containing meal was much greater than could be accounted for by the increase in lymph volume, and, like the increase in alkaline phosphatase, appears to represent a specific effect of ingested fat on the chemical composition of intestinal lymph. A parallel increase in the activity of these two enzymes has been found in the serum of alloxan-diabetic rats by Tuba and Hoare (12). The concentration of tributyrinase in the plasma is markedly diminished after external drainage of intestinal lymph for a 2 day period, suggesting that the intestine may normally be an important source of this enzyme for the plasma, as it has previously been shown to be for alkaline phosphatase.

SUMMARY

The amylase activity of intestinal lymph in the rat is generally less than that of plasma. The 24 hour secretion of this enzyme in the lymph is greater in fed than in fasting rats, but much of the increase is due to the increase in lymph volume. External drainage of this lymph for 2 days does not significantly alter the plasma level.

The tributyrinase activity of intestinal lymph is also less than in plasma. The 24 hour secretion in lymph is much greater in fed than in fasting rats and appears to represent a specific effect of ingested fat on the chemical composition of intestinal lymph. External drainage of the lymph markedly decreases the tributyrinase content of plasma.

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INSULIN METHYL ESTER

I. PREPARATION AND PROPERTIES

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There is considerable experimental evidence in support of the view that, in solution, the state of molecular dispersion of insulin is dependent on the net charge of the protein (10, 6, 8). In these investigations, alterations of the net positive charge were obtained by varying the pH of the solutions, with due consideration of the effect of the specific binding affinity of the protein for anions other than hydroxyl ions (8).

In the present investigation, an attempt was made to vary the net positive charge of the protein by chemical modification of specific ionizable groups. Among the various possibilities which exist, esterification of the carboxyl groups with acid methanol appeared most promising, since insulin is particularly resistant to denaturation by acids or alcohol.

The reaction of several proteins with acid methanol was studied by Fraenkel-Conrat and Olcott (7) who reported that, under specified conditions, complete esterification of the free carboxyl groups of proteins can be obtained without introducing other perceptible chemical modifications. This has been confirmed in the present work but it was found that in the case of insulin rigid control of the reaction with respect to time, temperature, and composition of the esterifying agent is of prime importance for the chemical, biological, and physical properties of the modified protein.

The present paper describes the preparation of insulin methyl ester and the effect of methylation on the titration curve, solubility, and biological activity of the protein, whereas in subsequent papers of this series the effect of methylation on the molecular-kinetic properties of the protein will be described.

EXPERIMENTAL

Acid methanol was prepared by passing dry hydrochloric acid gas into absolute methanol. After a suitable time interval, the acid concentration was determined by titration and the solution made up to the desired composition by the addition of absolute methanol. Concentrations varying between 0.1 and 0.01 N HCl were usually employed.

Weighed amounts of salt-free, desiccated, crystalline zinc insulin (Lilly)

were suspended in 100 ml. of acid methanol per gm. of insulin and incubated at the desired temperature for specified periods of time. In the presence of 0.1 N HCl the protein was initially insoluble in methanol and required mechanical stirring during the incubation period. In lower concentrations of acid, the protein dissolved spontaneously. The reaction was terminated by the addition of 2 to 3 volumes of ethyl ether, which caused almost complete precipitation of the hydrochloride of methyl insulin. The precipitate was centrifuged, dried with ether, and stored until used. Qualitative tests with dithizone indicated that during esterification zinc became detached from the insulin and remained in solution when the ester was precipitated.

Precipitation of insulin methyl ester from aqueous solution could be effected by adjustment of the pH to between 7 and 9 but this procedure was not applied routinely because of the danger of alkaline hydrolysis of the ester.

Methoxyl determinations were performed with the Zeisel method by the Elek Micro Analytical Laboratories, Los Angeles. Before analysis, the preparations were dissolved in water and dried from the frozen state in order to remove excess methanol (7).

Ammonia determinations were carried out on the supernatant alcoholic solution after precipitation of insulin methyl ester. 40 ml. of the acid methanol-ether solution were shaken with 10 to 15 ml. of water, in two portions, followed by 5 ml. of 0.01 N HCl. Because of the presence of traces of unprecipitated protein in this solution, ammonia was determined after vacuum distillation at pH 10, according to the directions of Archibald (1) and Speck (14). The ammonia gas was trapped in 0.5 to 1 ml. of boric acid and determined by titration with 0.1 N HCl, added from an ultramicro burette, with Tashiro's indicator for the determination of the endpoint. Since under these conditions of distillation glutamine is unreactive, it is unlikely that any detectable ammonia could have originated during the measurements from the amide N of the protein, which is more stable than that of glutamine (15). Indeed, direct nesslerization of the supernatant, after evaporation of ether and alcohol, gave much higher ammonia values than did the present method.

Results and Interpretation

Course of Esterification Reaction

The rate and extent of esterification of insulin were mainly studied under the following three experimental conditions: (a) in 0.1 N HCl at 25°; (b) in 0.1 N HCl at 0°; and (c) in 0.0125 N HCl at 25°. Less extensive experiments, conducted at other acid concentrations and temperatures, as well

as in the presence of small amounts of water, will not be reported, since no peculiarities were noted in these minor series of investigations. The principal results are represented in Fig. 1 in which the time of esterification is plotted along the abscissa and methoxyl content (absolute, and in per cent of the total carboxyl group) along the ordinate. Since 12,000 gm. of insulin contain approximately 12 moles of free carboxyl groups (2, 4, 9, 6), complete and exclusive esterification of these groups would correspond to a methoxyl content of 3.05 per cent —OCH₃. This value is reached after approximately 24 hours of esterification in 0.1 N HCl at
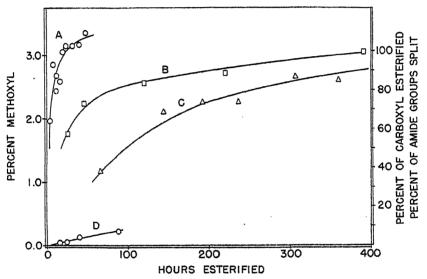
FIG. 1. Relation between the time of esterification of insulin and methoxyl content (Curves A, B, C). Curve D represents the per cent of amide groups split under the conditions represented by Curve A. The conditions of the experiments were as follows: Curves A and D, 0.1 N HCl in methanol, 25°; Curve B, 0.1 N HCl in methanol, 0°; Curve C, 0.0125 N HCl, 25°.

25°, whereas further treatment under these conditions leads to additional methoxyl uptake. Among the possible side reactions which could lead to the incorporation of an excess of methoxyl, the hydrolysis of protein amide groups appears most probable. Since the liberation of amide N would allow an equimolar amount of methoxyl groups to be incorporated, the liberation of ammonia occurring during esterification in 0.1 N HCl at 25° was determined (Fig. 1, Curve D). This is expressed as per cent of total amide groups (1.69 per cent (16)) of insulin. Although it appears that ammonia is continuously liberated during esterification, the amount

which is set free during the first 24 hours is negligibly small; *i.e.*, 0.027 mg. of NH₃ per 100 mg. of insulin, which is only about 2 per cent of the total amide nitrogen expressed as NH₃. However, upon prolonged esterification, the liberation of ammonia becomes appreciable and appears to be roughly equimolar with the amount of methoxyl in excess of that required for esterification of preexisting carboxyl groups. Much stronger secondary chemical changes were observed in the work of Kiesel and Znamenskaja (12) on the esterification of glycinin, but these authors used very high acid concentrations.

From the results plotted in Fig. 1 it is further seen that at lower acid concentrations (Curve C) or at lower temperatures (Curve B) the rate of esterification is greatly reduced. Since, as will be shown in another paper of this series, these latter conditions offer no perceptible advantage in the avoidance of those side reactions which reveal themselves by the presence of a second ultracentrifugal component, esterification was routinely performed for 24 hours in 0.1 N HCl at 25°.

Electrometric Titration Curves

In order to obtain information on the effect of esterification on the amphoteric properties of insulin, electrometric titrations on native and esterified insulin were performed. Measurements were carried out with the glass electrode, without exclusion of atmospheric carbon dioxide, on 120 mg. samples of protein present in 10 ml. of liquid. In most cases, the solution contained 0.1 M NaCl, and 1.0 N HCl was added from an ultramicro burette. Complications were introduced by the relative insolubility of insulin and its methyl ester within the pH range required for the titration of carboxyl groups (pH 2 to 6). Thus, insulin precipitates above pH 3.8 and an ester of approximately 3 per cent methoxyl content becomes markedly insoluble above pH 5.0. Although reliable evaluation of titration curves is possible only for measurements made in homogeneous solutions, it is of interest, nevertheless, to give a brief description of the nature of the anomalies encountered in the region of precipitation, particularly since in the classical study of the acid-base-binding properties of insulin by Harington and Neuberger (11) this problem has not been discussed.

In Fig. 2 the titration curve of native insulin is represented by (*a*) titration of an initially acid solution toward the alkaline side, and (*b*) titration in the reverse direction, as indicated by the arrows. In the pH range of precipitation the pH readings were repeated after every addition of acid or base till constant values were obtained. This required up to 15 minutes during the process of redissolution on the alkaline side of the isoelectric point. It is seen from the shapes of the curves of Fig. 2 that, when insulin precipitates on the acid side of the isoelectric point, a small, and variable,

inflection occurs, indicating some additional liberation of hydrogen ions in the process of precipitation. When the precipitate slowly redissolves on the alkaline side, strong hysteresis effects occur, dissolution being accompanied by the additional liberation of hydrogen ions. When the titration is conducted in the reverse direction, no hiatus is observed during precipitation on the alkaline side of the isoelectric point, but a deflection of the
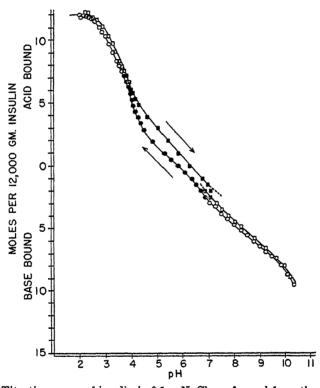
FIG. 2. Titration curve of insulin in 0.1 M NaCl, performed from the acid toward the alkaline side (□) and in the reverse direction (○). The filled symbols denote the region of precipitation. For further details, see the text.

curve shows redissolution on the acid side to be again connected with hysteresis. In the absence of sodium chloride, the curves show the usual clockwise displacement (3) and the phenomena just described occur at slightly different pH values, in accordance with the change in the pH range of precipitation. In the presence of 0.1 N potassium thiocyanate, the protein is precipitated within the entire acid range (18, 13). No inflections are found in the corresponding parts of the titration curve, but at alkaline

reactions dissolution is associated with the same phenomena as in the presence of sodium chloride. While limited data do not warrant a theoretical treatment of these results, it might be suggested that the effects of precipitation and dissolution on the titration curve may be correlated with the influence of the fixed charges in the precipitate on the hydrogen ion activity of the solution. When, for instance, precipitation is approached from the acid side, the molecules will arrange themselves so as to minimize the repulsions between positively charged groups. When a reaction alkaline to the isoelectric point is reached, there will now be an excess of negatively charged groups, some of which, being placed in close proximity to each other, will effect an additional binding of hydrogen ions. This effect will disappear and the pH will drop, when, during dissolution or during sufficient standing, the carboxyl groups will be free to rearrange themselves into random positions.

It is obvious that the results of titration in the region of precipitation cannot be utilized to judge the behavior of the carboxyl groups. However, a comparison between these groups in insulin and insulin methyl ester can be drawn from the observations made in pH regions acid to the precipitation zone.

Since the pK values of carboxyl groups in proteins vary within the wide limits of pK = 3.2 to 4.8 (5), the absolute number of these groups is indeterminate from such measurements and only relative comparison between insulin and its esters can be made. This has been done by comparing for various preparations the slopes of the titration curves at an arbitrarily selected value of pH 3.5.[1] The results of such measurements and calculations are plotted in Fig. 3. The relation between buffer capacity and methoxyl content is not linear, a finding also to be expected[1] if the α-carboxyl groups are esterified at a different rate than the side chain carboxyl groups of aspartic and glutamic acid. A buffer capacity of zero, indicating the total absence of titratable carboxyl groups, is reached at a composition corresponding to twelve methoxyl groups per 12,000 gm. of insulin, a value which, within the limits of experimental error, agrees closely with that corresponding to complete and exclusive esterification of the initially available carboxyl groups of insulin.

Esterification and Biological Activity

The biological activity of products of various degrees of esterification was determined and is given in Fig. 4, expressed as units per mg. of insulin. We are indebted to Dr. Edward D. Campbell of the Lilly Research Labo-

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[1] It is recognized that during the progress of esterification the pK values of the remaining carboxyl groups may change, owing to the change in the total surface charge. The chosen points of comparison at pH 3.5 are, therefore, not necessarily homologous for the different titration curves.

ratories for carrying out these biological assays. It is seen that esterification of approximately two-thirds of the initially available carboxyl groups has relatively little effect on the activity of the hormone but that the esterification of a relatively small additional increment of carboxyl groups rapidly diminishes biological activity.
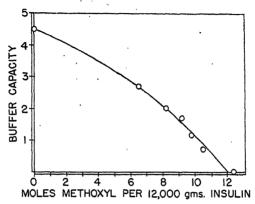
FIG. 3. The buffer capacity of insulin preparations of various degrees of esterification, at pH 3.5. For further details, see the text.
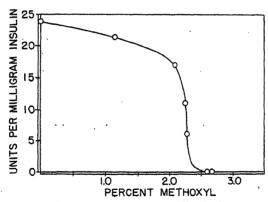
FIG. 4. A plot of the biological activity of insulin (units per mg.) *versus* the degree of esterification.

Saponification of the ester groups by alkali at low temperatures results in considerable reactivation of insulin. The conditions required for maximal recovery of activity have not been established.

Fibril Formation

When heated in acid solutions, insulin displays the phenomena of heat precipitation and fibril formation (17, 19). Qualitatively the same be-

havior was found in the present investigation for insulin methyl ester. The fact that insulin displays these effects only at strongly acid reaction might be regarded as evidence that unionized carboxyl groups are required. To test this contingency, both insulin and insulin methyl ester were heated between pH 2 and 4, according to Waugh's technique (19). As judged by the appearance of birefringence and by precipitation, insulin methyl ester required exactly the same high acidity as did native insulin, despite the fact that the charge relations of the ester at pH 4 are approximately the same as those of the unmodified insulin at pH 2. These experiments indicate that the specific intermolecular changes involved in fibril formation do not depend uniquely on the state of ionization of the carboxyl groups.

Miscellaneous Observations

When completely or nearly completely esterified insulin is titrated in water through the entire pH scale, a precipitate is formed at about pH 5.5 and redissolves at about pH 11. The latter value is not strictly reproducible, because the hysteresis effects described for insulin are also shown by the ester, and, moreover, some hydrolysis occurs at the alkaline reaction. In 0.1 M NaCl the precipitation zone is somewhat broader. In 0.1 M potassium thiocyanate, the ester is insoluble at all reactions below pH 11 to 11.5.

The ester is soluble in neutral and weakly acid methanol but only moderately soluble in strongly acid methanol (0.1 N HCl). The addition of 2 volumes of ethyl ether does not cause precipitation from neutral methanol but does so, as already described, from acid methanol.

SUMMARY

Incubation of insulin in acid methanol, under controlled conditions of temperature and of the concentration of hydrochloric acid, leads to an uptake of 12 moles of methoxyl groups per 12,000 gm. of insulin. Since under these conditions no significant amounts of ammonia are liberated, whereas the buffer capacity of the protein at approximately pH 3.5 has completely vanished, it has been concluded that the initially available carboxyl groups of insulin have been completely and exclusively esterified. More extensive treatment with acid methanol yields an additional incorporation of methoxyl groups, with the concomitant liberation of ammonia, presumably as a result of the partial hydrolysis of acid amide groups.

It has been shown that esterification of approximately two-thirds of the initially free carboxyl groups fails to reduce appreciably the biological activity of the hormone, whereas the incorporation of additional increments of methoxyl groups causes complete inactivation. Insulin methyl ester cannot be distinguished from insulin by qualitative observation of the phenomena of fibril formation but the two proteins differ markedly in solubility in aqueous solutions as well as in alcohol. As part of the present investigation, the anomalies encountered in the electrometric determination of titration curves of insulin were examined in detail.

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THE METABOLISM OF S³⁵-SODIUM ESTRONE SULFATE IN THE ADULT FEMALE RAT

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In general it is considered that conjugated estrogens are less active *in vivo* than the free form (1). This fact is attributed generally to the slow rate of hydrolysis of the conjugated form in the body or to an increased rate of excretion; however, it was shown recently (2) that pregnant and non-pregnant rats given S³⁵-sodium estrone sulfate by subcutaneous injection rapidly excrete S³⁵ as inorganic sulfate in the urine and feces, indicating that this conjugated estrogen underwent rapid hydrolysis in the body.·

More detailed data are presented in the present paper on the rate and possible pathway of excretion of the S³⁵ from S³⁵-sodium estrone sulfate given to adult female rats and the possible sites of hydrolysis of this conjugated estrogen.

EXPERIMENTAL

Rate of Excretion—Single doses of 0.5 mg. of sodium estrone sulfate,[1] labeled with S³⁵, in 1 ml. of 0.1 M phosphate solution, pH 7.45, were administered to each of eighteen adult female rats which were divided into groups of six. These rats were of the Sprague-Dawley strain and weighed from 200 to 250 gm. Administration of the radioactive estrogen was made via the subcutaneous, intravenous, or gastric route. The animals were placed in metabolism cages and fed *ad libitum*. Urine and feces were collected at 3, 6, 9, 12, 24, and 36 hour intervals.[2] These fractions were

[2] No detectable amount of free S³⁵ could be found after S³⁵-sodium estrone sulfate was incubated with urine under toluene for 12 hours at room temperature. Isolation of the S³⁵-sodium estrone sulfate by isotope dilution showed no loss of radioactive estrogen. Under similar conditions, S³⁵-sodium estrone sulfate incubated with fecal homogenates showed a production of only 5 to 10 per cent free S³⁵.

analyzed for their total S^{35} and inorganic S^{35} content by the method of Tarver and Schmidt (3). In the determination of the S^{35}-sodium estrone sulfate content of the urine, an aliquot was adjusted to pH 7.5, 25 mg. of non-labeled sodium estrone sulfate were added, and the isolation was carried out as described below. The S^{35}-sodium estrone sulfate content of the feces was determined as follows: A mixture of 1 part feces to 10 parts of water was homogenized in a Waring blendor for 3 to 5 minutes, 25 mg. of non-labeled sodium estrone sulfate were added, and the isolation was carried out as described later. All measurements of radioactivity were made by conventional counting methods. A 1.3 mg. per sq. cm. mica end window counter was used in the assay of radioactive samples. The results were corrected for decay and self-absorption. The average results of these experiments are shown in Fig. 1.

The recovery of S^{35} after 36 hours was 75, 86, and 86 per cent after administration by the intravenous, subcutaneous, and gastric routes, respectively.[3] The principal pathway of excretion of S^{35} varied with the method of administration of the radioactive estrogen. After the intravenous injection of the radioactive estrogen, 37 per cent of the S^{35} excreted was found in the feces and 63 per cent was found in the urine; however, after subcutaneous or gastric administration of the estrogen, 64 per cent of the S^{35} excreted was found in the feces and 36 per cent in the urine.

Of prime interest was the fact that all the S^{35} found in the urine and feces in these experiments was present as inorganic sulfate. No S^{35}-sodium estrone sulfate could be detected at any time interval in the urine or feces. This indicated that the conjugated estrogen underwent rather rapid hydrolysis in the body and that apparently the free sulfate was not utilized by the animal to any appreciable extent. The latter finding is essentially in agreement with that of Dziewiatkowski (4), who found that approximately 96 per cent of the S^{35} given by intraperitoneal injection in the form of sodium sulfate was excreted in the urine and feces in 120 hours. In his experiments, the major portion of the S^{35} was found in the urine.

Pathway of Excretion—As was pointed out above, a rather large percentage of the S^{35} released by hydrolytic cleavage of S^{35}-sodium estrone sulfate in the body was found in the feces. This observation prompted an investigation of the possible pathway for entry of the radioactive material into the intestine; hence, a study was made of the amount and the chemical form of any S^{35} that might be excreted via the bile of animals receiving this radioactive estrogen.

Bile fistulas were made by introduction of a cannula into the common

[3] Samples of blood taken 15 minutes after the intravenous injection of S^{35}-sodium estrogen sulfate contained very little radioactivity.

bile duct with a segment of a 23 gage needle. Small diameter plastic tubing carried the bile from the needle into a 4 ml. cylindrical plastic tube that was attached by skin sutures to the ventral surface of the rat. The distal end of the tube was equipped with a screw cap which permitted periodic withdrawal of bile. This method of bile sampling was advantageous in that it was unnecessary to immobilize the rats during the period of bile collection.

Bile fistula, female rats, 250 to 300 gm. in weight, were given single doses of 0.5 mg. of S^{35}-sodium estrone sulfate by intravenous or subcutaneous injection. The animals were placed in metabolism cages and were fed *ad libitum*. Urine, bile, and feces were collected up to 24 hours after the in-
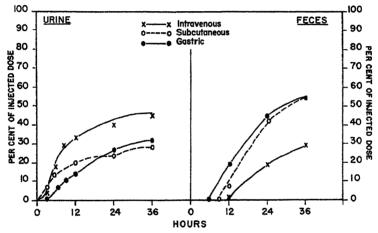
Fig. 1. Excretion of S^{35} in urine and feces of adult female rats given a single injection of 0.5 mg. of S^{35}-sodium estrone sulfate. Total counts of S^{35} per dose, 38,000 c.p.m.

jection of the estrogen and were analyzed for their total S^{35}, inorganic S^{35}, and S^{35}-sodium estrone sulfate.[4] Again all the S^{35} present was found to be in the inorganic form. The distribution is shown in Table I. After subcutaneous or intravenous injection of S^{35}-sodium estrone sulfate, the amount of S^{35} found in the bile was approximately 10 per cent of the total S^{35} excreted in 24 hours. With subcutaneous injection this represents approximately 25 per cent of the total S^{35} found in the bile and feces, and with intravenous injection, approximately 15 per cent of the total S^{35} in the biliary and fecal excretions. It will be noted that in the bile fistula

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[4] Incubation of S^{35}-sodium estrone sulfate with bile for 6 hours at 37° caused no release of free S^{35}. In these animal experiments the interval of time between collections of bile samples for assay was no greater than 6 hours.

rats subcutaneous injection of the labeled estrogen resulted in a high urinary excretion of S^{35}, which could not be resolved with the distribution found in normal rats.

The results of these experiments indicate that the bile does not serve as the main excretory pathway for the S^{35} found in the feces of animals given S^{35}-sodium estrone sulfate by intravenous or subcutaneous injection. The exact site of entry of the S^{35} into the intestine is not known. The S^{35} which does enter the bile has already been cleaved from the estrogen.

Hydrolytic Action of Organ Homogenates—It was of interest to investigate the possible sites of hydrolysis of sodium estrone sulfate in the body.

TABLE I

24 Hour Excretion of S^{35} in Bile Fistula, Female Rats Given Single Injection of 0.5 Mg. of S^{35}-Sodium Estrone Sulfate

Total counts of S^{35} per dose, 38,000 c.p.m.

Experiment No.	Route of administration	Urine		Bile		Feces		Total recovery	
		Counts S^{35} per min.	Per cent S^{35} excreted	Counts S^{35} per min.	Per cent S^{35} excreted	Counts S^{35} per min.	Per cent S^{35} excreted	Counts S^{35} per min.	Per cent injected dose
I	Subcutaneous	16,000	53	2900	10	11,500	37	30,400	80
II	"	20,800	57	5200	14	10,200	29	36,200	95
III	"	14,700	53	2400	9	10,400	38	27,500	73
IV	Intravenous	14,800	57	1900	7	9,100	36	25,800	68
V	"	14,600	43	1570	5	15,700	52	31,900	84
VI	"	14,700	48	1730	6	14,300	46	30,730	80
VII	"	19,760	60	2940	9	9,900	31	32,600	86

Consequently, a study of the hydrolytic activity of various organ homogenates on S^{35}-sodium estrone sulfate was carried out.[5] The organs used in this study were liver, pancreas, small intestine, large intestine, ovary, uterus, adrenal, and kidney. The amount of S^{35}-sodium estrone sulfate remaining after incubation with the homogenates was determined by the "inverse" isotope dilution technique (5). The procedure used in these studies is described below.

Adult female white rats of the Sprague-Dawley strain, 200 to 250 gm. in weight, were anesthetized with ether and the blood was removed by severance of the jugular veins. The desired organ was removed immediately and was homogenized in distilled water at 4° in a Waring blendor or

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[5] No production of free S^{35} was noted when S^{35}-sodium estrone sulfate was incubated with plasma for $2\frac{1}{4}$ hours at 37°.

a glass homogenizer tube; both methods gave the same results. The amounts of organ were chosen so that the final homogenate contained 20 mg. of wet tissue per ml. This homogenate was centrifuged at 10,000 r.p.m. in a Servall angle centrifuge for 30 minutes at 4°. The supernatant contained over 80 per cent of the enzymatic activity of those organ homogenates which effected a hydrolysis of the conjugated estrogen. Equal volumes of the supernatant and 0.1 M PO_4 buffer, pH 7.45, were mixed and were tested for activity as follows: 10 ml. of the buffered homogenate were transferred to a 50 ml. glass-stoppered Erlenmeyer flask, and 2.0 mg. of S^{35}-sodium estrone sulfate, specific activity 76,000 c.p.m. per mg., were added. The flask was stoppered tightly, and the contents were mixed by shaking. The flask was then placed in a water bath at 37° for $2\frac{1}{2}$ hours.

The following controls were run on each homogenate: (a) 2.0 mg. of S^{35}-sodium estrone sulfate in 10 ml. of 0.1 M PO_4 buffer, pH 7.45, incubated at 37° for $2\frac{1}{2}$ hours, (b) the same amount of substrate in 10 ml. of buffered boiled homogenate, pH 7.45, incubated at 37° for $2\frac{1}{2}$ hours, and (c) 2.0 mg. of S^{35}-sodium estrone sulfate in 10 ml. of buffered homogenate which were not incubated but extracted immediately after mixing by the procedure described below.

At the end of the incubation period, 100 mg. of non-radioactive sodium estrone sulfate were added to the incubation mixture. The contents were mixed, and immediately thereafter 35 ml. of n-butanol were added. The flask was stoppered and the contents were mixed by vigorous shaking for 2 to 3 minutes. Inasmuch as an emulsion always formed, it was necessary to centrifuge the mixture at 3000 r.p.m. for 15 to 20 minutes at room temperature in order to break the emulsion. The butanol-soluble fraction was removed and, if necessary, the non-butanol-soluble residue was reextracted a second time with a fresh change of butanol.

The butanol extracts were transferred to a 125 ml. separatory funnel and washed successively three times with 1.0 ml. portions of N NaOH and three times with 1.0 ml. portions of water. After the final washing, the butanol fraction was evaporated to dryness in vacuo and then was taken up in a small volume of warm absolute methanol, and warm anhydrous ether was added slowly till a slight turbidity had developed. The mixture was allowed to stand overnight at 4°. The crystals were collected on a dry filter and then dried over P_2O_5. It was necessary to recrystallize this material three or four times from this same solvent. Usually this sufficed to bring the specific activity and melting point of the material to a constant value. The sodium estrone sulfate was recrystallized from n-butanol, but usually this did not change the specific activity or melting point of the compound. M.p. 226–228° (uncorrected). Admixture with pure sodium estrone sulfate did not depress the melting point.

$C_{18}H_{21}O_5S \cdot NaH_2O$.   Calculated.   C 55.21, S 7.92, H 6.01

                  Found.   " 55.10, " 8.01, " 5.92

The total S^{35} content and the specific activity of the sodium estrone sulfate were determined. The amount of unchanged S^{35}-sodium estrone sulfate remaining in the incubation mixture was calculated by the "inverse" isotope dilution technique (5).

The results of these experiments showed that under the conditions used only the liver homogenate contained an enzyme system capable of hydrolyzing the sulfate ester of estrone. In $2\frac{1}{2}$ hours at 37° the extract from liver was able to release completely the sulfate from sodium estrone sulfate. In the case of the other organ homogenates, less than 10 per cent free sulfate could be detected under similar conditions. None of the controls showed any significant loss of S^{35}-sodium estrone sulfate or the production of any free sulfate.

All the S^{35} released from the S^{35}-sodium estrone sulfate by liver homogenate was inorganic in nature. This was ascertained as follows, after it was shown that there is no S^{35}-sodium estrone sulfate remaining in the liver homogenate: 20 mg. of non-labeled sodium sulfate were added to the incubation mixture and then 5 ml. of 10 per cent $Cl_3C \cdot COOH$; the mixture was filtered and all the S^{35} was precipitated as benzidine sulfate and the total S^{35} content was determined in the usual way. A control, containing S^{35}-sodium estrone sulfate and buffer and treated in a similar manner, showed that less than 10 per cent of the S^{35} was released from the conjugated estrogen.

It is particularly interesting that liver homogenate is capable of effecting the hydrolysis of this conjugated estrogen. As was pointed out by Szego and Roberts (6), the ratio of free to conjugated estrogen is much lower in the blood than that in the bile. In addition, Pearlman *et al.* (7) found that the gall-bladder bile of pregnant cows contained free estrone as the major estrogen. On the basis of the results presented in the present paper, it is evident that the liver contains an enzyme system capable of hydrolyzing the sulfate ester of estrone. Thus, at least in part, the estrone in the bile could be derived from the estrone sulfate in the blood.

SUMMARY

The metabolism of a conjugated estrogen, S^{35}-sodium estrone sulfate, in the adult female rat was studied.

Over 75 per cent of the S^{35} of the radioactive estrogen given to female rats by intravenous, subcutaneous, or gastric injection was found in the urine and feces in 36 hours. All the excreted S^{35} was inorganic in nature.

Administration of S^{35}-sodium estrone sulfate by subcutaneous or intravenous injection to bile fistula, female rats resulted in the excretion of less

than 15 per cent of the S^{35} in the bile; the remaining S^{35} was found in the urine and feces. Again all the S^{35} excreted was inorganic in nature.

Various organ homogenates were examined for enzyme systems capable of hydrolyzing this conjugated estrogen. Liver homogenate was the only one examined that was capable of effecting a hydrolysis of this ester.

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